Entamoeba histolytica Cysteine Proteinases Facilitate Parasite Invasion of the Colon by Disrupting the Colonic Mucus Barrier

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Abstract

The protozoan parasite *Entamoeba histolytica* is the etiological agent of human amebiasis. Trophozoites colonize the colonic mucus layer and may invade the epithelium subsequent to overcoming the mucus barrier. MUC2 is the major gel-forming mucin secreted by goblet cells in the colon and serves to maintain epithelial barrier function as well as acting as a major host defense against invading pathogens. The polymerization of MUC2 monomers via the N- and C- terminal cysteine rich D-domains is essential for mucus gel formation and confers protection to the underlying mucosa. Amoebae secrete cysteine proteinases, glycosidases and an unidentified mucus secretagogue, which may play a role in overcoming the protective mucus barrier. We hypothesize that *E. histolytica* cysteine proteinases as well as glycosidases are involved in mucus degradation and weakening of the mucus barrier by disrupting mucin polymerization. Amoebae secreted cysteine proteinases were shown to degrade the cysteine rich regions of MUC2 involved in polymerization and abrogate its protective function. More importantly, the major *E. histolytica* surface proteinase, cysteine proteinase 5 (EhCP5) was shown to specifically degrade [^{35}S]cysteine labeled colonic mucin as effectively as secreted components. Moreover, trophozoites genetically engineered to express low levels of CP activity were incapable of traversing a mucus barrier and destroying the underlying epithelium, indicating a strong dependence between amebic invasiveness and cysteine protease activity. In addition, we have demonstrated that EhCPs specifically target the MUC2 C-terminus resulting in destabilization of the mucin polymeric network. Parasite glycosidase activity was also shown to contribute to mucin oligosaccharide degradation. Taken together, these results indicate that *E. histolytica* can substantially weaken the colonic mucus barrier via proteolytic degradation and glycosidase activity to compromise the gel and allow the parasite to invade the underlying colonic epithelium. These findings have made a major contribution to our understanding of how *E. histolytica* virulence factors interact with innate defenses of the gut in the of pathogenesis of intestinal amebiasis. This information is necessary to devise molecular and immunological approaches in the treatment of intestinal amebiasis.

Abrégé

Le parasite protozoaire *Entamoeba histolytica* est l'agent étiologique de l'amibiase humaine. Les trophozoites colonisent la couche muqueuse du colon et peuvent envahir l'épithélium après avoir passer la barrière muqueuse. MUC2, la mucine qui forme un gel, est la mucine principale sécrétée par les cellules de goblet dans la colon. MUC2 sert à maintenir la fonction de barrière épithéliale et sert aussi comme première défense contre les pathogènes envahissants. La polymérisation des monomères de MUC2 via les terminus N et C, contenant des domaines D riches en cystéines, est essentielle pour la formation du gel muqueux et la protection des muqueuses sous-jacentes. Les amibes sécrétent des protéinases à cystéine, des glycosidases et une sécrétagogue à mucus non-identifiée. Tous ces éléments pourraient aider le parasite à franchir la couche protectrice de mucus. Nous hypothésons que les protéinases à cystéine et les glycosidases de *E. histolytica* sont impliquées dans la dégradation des mucines et donc dans l'affaiblissement de la couche protectrice de mucus via la désintégration des polymères de mucine. Les protéinases à cystéine sécrétées par les amibes ont démontré l'abilité d'abolir la fonction protectrice de MUC2 par la dégradation des régions riches en cystéines nécessaires à la polymérisation. De plus, la protéinase majeure de *E. histolytica* exposée à la surface du parasite, la protéinase à cystéine 5 (EHCP5), a pu spécifiquement dégrader des mucines coloniques marquées avec de la [^{35}S]-cystéine. Cette dégradation par la EHCP5 était comparable à celle observée par les produits sécrétés du parasite. Des trophozoites génétiquement modifiés pour exprimer de bas niveaux d'activité CP, étaient incapables de traverser la couche protectrice de mucus et de détruire l'épithélium sous-jacent, indiquant une forte corrélation entre l'activité des protéinases à cystéine et l'invasion amibienne. Nous avons aussi démontré que les EHCPs ciblent spécifiquement le C-terminus de MUC2, provoquant la déstabilisation du réseau polymérique de mucines. Nous avons montré que les glycosidases du parasite contribuent aussi à la dégradation des oligosaccharides de mucine. Pris ensemble, ces résultats indiquent que *E. histolytica* peut significativement affaiblir la couche protectrice de mucus colonique via une dégradation protéolitique et une activité glycosidase qui compromettent le gel et permettent au parasite d'envahir l'épithélium sous-jacent. Ces trouvées nous aident

grandement à mieux comprendre comment l'intéraction entre les facteurs de virulence de *E. histolytica* et les défenses innées de l'intestin contribue à la pathogénèse de l'amibiase intestinale. Cette information est nécessaire pour concevoir de nouvelles approches moléculaires et immunologiques afin de traiter l'amibiase intestinale.

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Finally, I must thank my parents and family for supporting me both emotionally and financial during my studies, and my husband for all his support and patience.

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List of Abbreviations

ALA, amebic liver abscess

AP-A, amoebapore A

BSA, bovine serum albumin

CHO, chinese hamster ovary

CRD, carbohydrate recognition domain

E-64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane

EdCP, *Entamoeba dispar* cysteine proteinase

EhCP, *Entamoeba histolytica* cysteine proteinase

EDTA, ethylenediaminetetraacetic acid

ER, endoplasmic reticulum

G418, G-418 sulfate, Geneticin

Gal-Lectin, galactose-inhibitable adherence lectin

Gal, galactose

GFP, green fluorescent protein

GalNAc, N-Acetyl-D-galactosamine.

GlcNAc, N-Acetyl-D-glucosamine

GalNAc-T, polypeptide GalNAc transferase

GI, Gastrointestinal

h, hour

HEPES, 4-2-hydroxyethyl-1-piperazineethanesulfonic acid

IL-1, interleukin 1

IL-1β, interleukin 1 beta

pIL-1β, preinterleukin 1 beta

IL-8, interleukin 8

kDa, kilodalton.

M_r, molecular mass

MT, microtubule.

MW, molecular weight.

PBS, phosphate buffered saline

PGE_2, prostaglandin E2

PKC, protein kinase C

PSC, Pefabloc SC

PSM, porcine submaxillary mucin

S4B, Sepharose 4B

SCID-HU-INT, SCID-human intestine

SD, standard deviation

SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis

SP, secreted products

TNF-α, tumor necrosis factor alpha

TR, tandem repeat

VNTR, variable number tandem repeat

vWF, von Willebrand factor

Z-Arg-Arg-pNA, benzyoxycarbonyl-L-arginyl-L-arginine-paranitroanilide

Thesis Office Statement

As an alternative to the traditional thesis format, the dissertation can consist of a collection of papers of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research.

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis.

The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges preceding and following each manuscript are mandatory.

The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation". The thesis must include the following: (1) a table of contents: (2) a brief abstract in both English and French (3) an introduction which clearly states the rational and objectives of the research: (4) a comprehensive review of the literature (in addition to that covered in the introduction to each paper) (5) a final conclusion and summary: (6) a thorough bibliography and (7) an appendix containing an ethics certificate in the case of research involving human or animal subjects, microorganisms, living cells, other biohazards and/or radioactive material.

In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.

Statement of Originality

The following aspects of this thesis are considered contributions of original knowledge:

Manuscript I

This is the first study to identify and characterize *E. histolytica* secreted cysteine proteinases as the major parasite virulence factor involved in degrading colonic mucin in vitro. More importantly, the cytoprotective functions of the degraded mucin were shown to be diminished.

Manuscript II

Using antisense technology, this is the first study to show a direct correlation between *E. histolytica* cysteine proteinase activity and colonic mucin degradation. In addition, we have shown that the parasite utilizes cysteine proteinases to overcome the protective mucus barrier prior to invading colonic epithelial cells.

Manuscript III

This is the first report to determine the mechanism used by *E. histolytica* to disrupt the mucin polymeric network. We demonstrated that the C-terminus of MUC2 is targeted by the cysteine proteinases, and identified the cleavage site involved in breaking the MUC2 polymer. This is the first study to characterize how an enteric pathogen compromises mucin at the structural level.

Manuscript IV

We have demonstrated that *E. histolytica* glycosidases degrade colonic mucin oligosaccharides. This is the first report in which these enzymes are identified as potential virulence factors involved in amebic invasion.

Statement of Authorship

This thesis consists of four manuscripts co-authored with my supervisor Dr. Kris Chadee. Dr. Chadee provided financial support for the laboratory work, advice with regards to experimental design and corrections of the thesis and manuscripts. Kathy Keller provided technical support in the papers in which she is co-author. Drs. David Mirelman and Serge Ankri provided the pSA8 and pEhAct-Neo plasmids and helpful discussions for use in Manuscript II. Dr. Gunnar Hansson and Martin Lidell provided the MUC2 recombinant proteins, discussions and technical advice for Manuscript III.

Section I: Literature Review

Introduction

The protozoan parasite *Entamoeba histolytica* is the etiological agent of human amebiasis. Approximately one percent of the world's population is infected with the parasite and about 10% of those infected develop invasive disease [1]. *E. histolytica* is a major cause of morbidity and mortality in developing countries throughout the world and infection rates are among the highest in areas such as Mexico, India, sub-Saharan Africa and Asia. The major symptoms of the disease are bloody or mucoid containing stool, diarrhea, colitis, liver abscess formation, and subsequent death if left without treatment.

The initiating events contributing to invasive disease are ill defined and very few studies have focused on the initial interactions between the parasite and the innate host defense of colonic mucin. Following excystment in the small intestine, *E. histolytica* trophozoites colonize the mucus layer of the colon by adhering to galactose and N-Acetyl-D-galactosamine residues present on mucin via a 170 kDa Gal/GalNAc lectin [2]. In most cases, the parasite remains in or on the mucus layer as a harmless commensal and forms cysts that are passed out in the feces to continue the lifecycle. However, in a small percent of cases, the parasite overcomes the mucus barrier, makes contact with the underlying epithelium and causes cytolysis of host epithelial and inflammatory cells. The trophozoites then migrate through the mucosa and degrade tissues and extracellular matrix proteins via the action of secreted cysteine proteinases [3]. Finally, during migration, the parasites may be picked up by the circulatory system and passively disseminate to organs such as the liver where they become lodged and form an abscess.

The parasite virulence factors involved in mucus disruption prior to epithelial cell invasion have not previously been identified and cysteine proteases are likely key molecules responsible for depleting the mucus barrier. *E. histolytica* constitutively secretes copious amounts of cysteine proteases and these have potent lytic activity against a range of host proteins such as collagen [3], fibronectin [4], complement [5], and immunoglobulins. In addition, the parasite produces glycosidases that may contribute to

mucus degradation [6]. Mucus secretion is also induced during infection by an unknown secretagogue, possibly contributing to depletion of the mucus barrier.

MUC2 is the major gel forming mucin secreted by goblet cells of the colon. The MUC2 molecule is composed of two mucin domains, which are heavily glycosylated with *O*-linked oligosaccharides and are resistant to proteolytic attack. The N- and C- terminal regions flanking the mucin domains are rich in cysteine residues and are involved in polymerization of MUC2 with corresponding termini [7]. These regions are poorly glycosylated in comparison to the mucin domains and may be susceptible to proteolytic attack. The intrinsic gel-forming and protective properties of mucus are dependent upon both the oligosaccharide's ability to protect the protein core, as well as the molecule's ability to form polymers via the poorly glycosylated regions. Alterations in the mucin polymeric network such as depolymerization would likely destabilize the mucus gel and contribute to weakening of the barrier. This depletion could facilitate parasite invasion of the colon by allowing the trophozoites to overcome the mucus barrier. **The objective of this study was to identify the *E. histolytica* enzymes that facilitate parasite invasion of the colon by disrupting the mucin polymeric network.** The specific aims were:

1) ***To characterize E. histolytica proteinase activity against colonic mucin***
2) ***To determine the specific role cysteine proteinases play in mucin degradation and epithelial cell invasion***
3) ***To identify the specific cysteine proteinase cleavage sites on MUC2***
4) ***To characterize glycosidase activity by amebae secreted components***

These studies have identified an important *E. histolytica* virulence factor, the cysteine proteinases, as being the main class of enzymes responsible for destabilizing the mucin polymeric network and allowing the parasite to traverse the mucus barrier. In addition, parasite glycosidase activity and mucus secretagogue activity may also play secondary roles in mucus depletion. These findings have made a major advance in our

understanding of how *E. histolytica* cysteine proteinases overcome luminal barrier function in intestinal amebiasis.

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Chapter 1: *Entamoeba histolytica*: life cycle and virulence factors

1.1 Introduction to *Entamoeba histolytica*

Entamoeba histolytica is a protozoan parasite, and the etiological agent of human amebiasis. It is estimated that one percent of the world's population is infected with the parasite of which 10% develop amebic colitis and/or extraintestinal disease [1]. The parasite causes an estimated 100,000 deaths per year and is the third leading cause of morbidity and mortality due to a parasitic disease in humans after malaria and schistosomiasis [2]. Amebiasis affects mainly people in developing countries where sanitation conditions are poor, but increased international travel has caused the parasite to be of concern in developed nations. Infection with *E. histolytica* can be very distressful to the host and may cause abdominal pain, severe diarrhea as well as fever, bloody mucoid stool, extraintestinal liver abscess and death if left untreated [3].

E. histolytica belongs to the Phylum protozoa, Class Lobosea, Order Amoebida and family Entamoebidae. Initial attempts to identify the parasite have proven difficult due to the presence of other nonpathogenic amoebae in the normal human colon. The organism was first identified in 1875 by Fedor Lösch, a Russian physician, and was initially called "Amoeba coli" by its discoverer. It was not until several years later in 1903 that Fritz Schaudinn created the species name *Entamoeba histolytica.* At that time, scientists believed that the parasite could exhibit varying degrees of virulence since many people infected with what was once thought of as *E. histolytica*, could spontaneously clear their infection and never develop disease. This parasite's alter ego made studies of the pathogenesis of the disease very problematic. There were no clear morphological differences found between the "pathogenic" and "nonpathogenic" amoebae, therefore they were believed to be one of the same. In 1925, Emile Brumpt suggested that there were two species, one, which causes disease and has the potential to invade, and the other which would never cause disease, named *Entamoeba dispar.* It was not until later in the 1970s and 1980s with the development of new biochemical and genetic data that there was enough evidence to support the existence of *E. dispar* as a separate species. In 1993 a formal

redescription of *E. histolytica* was published which distinguished it from *E. dispar* [4]. Thanks to this differentiation, future research on the pathogenesis of amebiasis has shifted towards discovering the genetic and biochemical differences between these two organisms with regards to potential virulence factors present in *E. histolytica,* which may be absent or in lower abundance in the non-invasive commensal *E. dispar*. Since its redescription, many virulence factors unique to *E. histolytica* have been shown to be involved in the pathogenesis of invasive amebiasis.

1.2 *E. histolytica* Life Cycle and Cell Biology

E. histolytica is a parasite with a simple life cycle. Humans become infected by ingesting the infective cyst form of the parasite with fecally contaminated food and/or water. An *E. histolytica* cyst contains four nuclei and measures about 12 μM in diameter and this resistant stage of the parasite can survive outside the host for several days in a moist environment. Upon ingestion, the cyst passes through the stomach and small intestine and excystation occurs in the terminal ileum or as the cyst enters the large intestine, followed by a series of nuclear then cytoplasmic divisions. This results in the release of eight uninucleated trophozoites into the colon. The trophozoite is not infective and cannot survive passage through the harsh environment of the stomach. Trophozoites range in size from 7 to 30 μm in diameter and reside in or on the mucus layer of the large bowel where they feed on bacteria and divide by binary fission. In asymptomatic cases, the amoebae will colonize the large intestine as harmless commensals, aggregate in the intestinal mucus layer and form cysts. However, in a small percent of cases, the parasite crosses the mucus barrier, and binds and lyses host epithelial cells via the galactose and N-Acetyl-D-galactosamine inhibitable adherence lectin (Gal-lectin) and invades the colonic epithelium. During invasion, the trophozoite may be carried away by the host's circulatory system to distant soft tissue organs such as the liver, lungs, or brain and causes abscess formation.

E. histolytica was considered a primitive eukaryotic cell since its cytoplasm seemed to be lacking some characteristic membrane bound organelles such as mitochondria,

Golgi apparatus, rough endoplasmic reticulum, centrioles and microtubules. However, there is now abundant genetic and biochemical evidence which supports the existence of functionally related organelles [5-11]. The cytoplasm of the trophozoite does contain a dense population of acidic vacuoles involved in the endocytic process. Parasite food vacuoles can often be seen filled with starch or bacteria in amoeba from xenic cultures or containing ingested red blood cells when recovered from patients with amebic dysentery.

1.3 Epidemiology and Treatment of *E. histolytica* Infection

In the past, up to 90% of *E. histolytica* infections reported in humans were actually intestinal colonization with *E. dispar* and epidemiological studies involving the identification of *E. histolytica* by examination of stool samples were not accurate. Today there are reliable diagnostic tests available to identify *E. histolytica* infection. Currently, identification by microscopic analysis of stool alone is not sufficient for diagnosis, but in conjunction with serological/fecal testing and in some cases isoenzyme characterization, an accurate diagnosis of *E. histolytica* infection can be made. In addition, the development of reliable PCR based detection methods have proven valuable due to the specific and sensitive nature of these types of tests [12, 13].

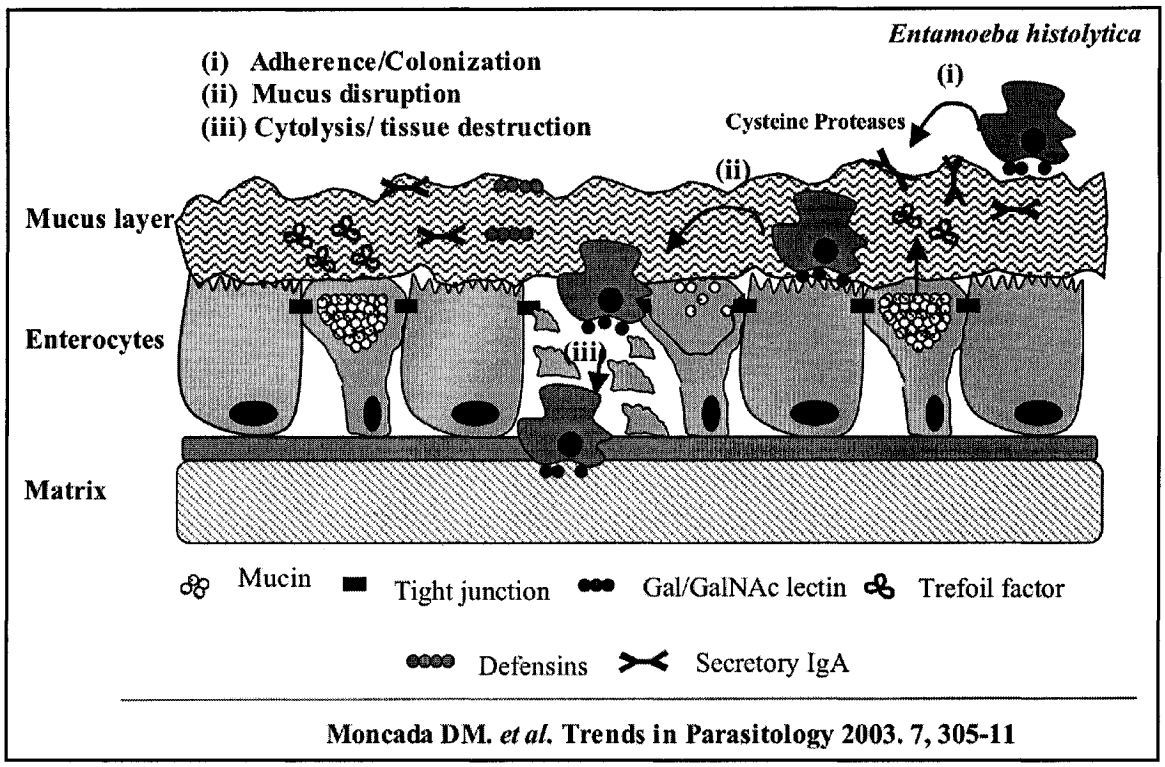
Infection with *E. histolytica* can be found throughout the world, although endemic areas in developing countries such as Mexico, South America, Sub Saharan Africa, India and Pakistan have the highest infection rates [14-16]. Non-endemic areas including the United States and Canada have infection rates of approximately 1-2%. Risk groups in developed countries include travelers to or recent immigrants from developing countries, as well as residents of mental institutions [17-19], sexually active homosexuals [20-24], lower socioeconomic groups in the southern United States [25] and immuno-compromised individuals [26, 27]. Interestingly, the pandemic of HIV infection has not resulted in an increase in invasive amebiasis although colonization may be common [3] [28].

Treatment of *E. histolytica* infection may differ for noninvasive and invasive infection, and the drugs used to treat the disease are not necessarily approved in all countries. Non-invasive infections are often treated with paromomycin or diloxanide furoate, which is not available in the United States. Treatment for invasive infection is mainly by the use of nitroimidazoles such as metronidazole (Flagyl) or other effective compounds with longer half-lives such as tinidazole (not yet available in the USA or Canada) [29]. To date, metronidazole is the drug of choice for use in patients with acute and chronic forms of invasive amebiasis and drug resistance is not yet a major problem but should be of some concern due to its world-wide usage and the ability to generate metronidazole resistant parasites in culture [30]. A mucosal vaccine against the parasite would be a useful alternative and is currently a topic of study by members of our laboratory [31].

1.4 Pathogenesis of Invasive Amebiasis

Pathogenesis is defined as the mechanisms involved in the initiation, evolution, and subsequent outcome of a disease and involves both host and parasite factors. There are at least three separate and distinct phases in the pathogenesis of invasive amebiasis and these are illustrated in **Fig. 1.1**. The first stage is (i) colonization of the colonic mucus layer by the parasite Gal-lectin. Following colonization, the parasite (ii) disrupts and causes depletion of the protective mucus barrier and gains access to and (iii) binds host epithelial cells using the Gal-lectin. The invading trophozoites then proceed to kill host epithelial and inflammatory cells in a contact-dependent fashion, inducing severe colonic ulceration. During invasion of the host tissue, the parasite may be swept away by the circulatory system and become lodged in distant organs, most notably the liver, where amebic abscesses may form. There are four clinical manifestations of invasive intestinal amebiasis, all of which are generally acute: bloody diarrhea or dysentery, fulminating colitis, amebic appendicitis, and ameboma of the colon [32]. Diarrhea and dysentery are usually the main symptom in about 90% of invasive infections.

Figure 1.1 Schematic Diagram of Invasive Amebiasis.



Colonization of the colon is mediated by adherence of the parasite to galactose and N-acetyl-D-galactosamine residues of mucin oligosaccharides via the surface Gal-lectin. *E. histolytica* Gal-lectin is a heterodimeric protein consisting of a 170 kDa heavy subunit disulfide linked to a 31/35 kDa light subunit, which noncovalently associates with a 150 kDa intermediate subunit [33]. The parasite binds exposed terminal galactose/N-acetyl-D-galactosamine residues present on mucin as well as target epithelial cells [34, 35]. Purified rat and human colonic mucin act as the main receptors for the parasite and the Gal-lectin has been shown to bind these glycoproteins with high affinity (K_d=8.2 × 10^{-11} M^{-1}) [36]. In addition, colonic mucin has a cytoprotective function and has been shown to inhibit adherence of the parasite to target epithelial cells in vitro [37]. Interestingly, although *E. histolytica* cannot successfully colonize the rat colon, purified rat colonic mucin is very effective at inhibiting adherence of the parasite to epithelial cells [37]. This supports the universal

role of mucin carbohydrates in acting as receptors for enteric organisms and also indicates that there are additional host and/or parasite factors involved in colonization.

In asymptomatic cases of amebiasis, the parasite remains in the mucus layer causing no harm to the host, and will continue out its life cycle and form infectious cysts that are passed out in the feces. However, in cases of invasive disease, the parasite overcomes the innate host defense of the colonic mucus barrier and causes a depletion of the mucus layer by mechanisms that are not well defined and are currently under investigation. Previous studies using the gerbil model of invasive amebiasis have revealed that this depletion may be a result of parasite-induced hypersecretion of goblet cell mucus [38]. Increased mucus release and goblet cell cavitation have been observed prior to invasion of the colonic mucosa in the same model [39]. Currently, there is no consistent animal model for intestinal amebiasis. The gerbil model has many limitations, and trophozoites grown in culture are not capable of colonizing or invading the gerbil cecum.

E. histolytica trophozoites have been shown to evoke massive mucus secretion in human colonic epithelial cells through a Protein kinase C-dependent mechanism [40]. In addition, a cyclooxygenase-like enzyme has been isolated from the parasite and characterization studies have revealed that the enzyme catalyzes the conversion of arachidonic acid into prostaglandin E_2 (PGE_2) [41]. The enzyme is constitutively expressed by amoebae and PGE_2 is known to act as a potent mucin secretagogue in both human colonic epithelial cells and rat colonic loop studies [42]. Although to date, the parasite secretagogue has not been identified, parasite production of PGE_2 likely contributes to invasion of the colonic epithelium by inducing the release of goblet cell mucin. In addition to inducing hypersecretion of high molecular weight mucin from goblet cells, *E. histolytica* secretagogue activity also causes the release of abundant low molecular weight proteins that have not been characterized [43].

Although hypersecretion of mucus from goblet cells may be at least one factor involved in parasite invasion, it is not the sole event involved in the removal of the

colonic mucus layer by the parasite. *E. histolytica* trophozoites release abundant amounts of cysteine proteinases [44, 45] as well as glycosidases [46, 47] into culture medium. These enzymes may act in a concerted fashion to disassemble the mucin polymer and prevent the parasite from becoming trapped and sloughed off with the mucus layer. β-N-acetylhexosaminidase is the major glycosidase released by the parasite [48], in addition, significant levels of α-D-glucosidase, β-L-fucosidase, β-D-galacosidase and β-D-glucosidase activities have been found in parasite conditioned medium. These enzymes have not been directly implicated in the pathogenesis of invasive amebiasis but their contribution towards degrading mucin oligosaccharides still needs to be investigated. A previous study has reported that *E. histolytica* cellular lysates and secreted products are ineffective at degrading mucin, but the methods used to analyze the degradation products may not have been sensitive enough to detect small changes in such a large molecule [46].

Following disruption of the mucus layer, the parasite is able to gain access and bind to the colonic epithelium. Trophozoites efface the enteric microvilli to establish intimate contact with enterocytes by a mechanism involving the disruption of the actin-bundling protein villin [49]. Once the parasite makes contact, the trophozoite must overcome additional components of the epithelial barrier. Epithelial tight junctions physically link neighboring cells of the intestinal tract to one another. These junctions regulate barrier function by forming a seal that selectively controls the paracellular transport of molecules between epithelial cells [50]. The junctional complex also maintains the polarity of the epithelium by separating the apical and basolateral domains of the plasma membrane. *In vitro* studies have shown that *E. histolytica* disrupts epithelial barrier function upon contact with the epithelium by causing a rapid drop in the transepithelial electrical resistance of confluent monolayers which is a measurement of tight junction integrity [51]. In addition, the trophozoites cause an increase in paracellular permeability, as measured by the abnormal passage of small molecules such as [^{14}C]mannitol between epithelial cells. During invasion, the parasite may physically migrate between epithelial cells, and this event would likely contribute to disruption of the tight junction complex. The events involved in tight

junction alterations induced by the parasite are poorly understood, although dephosphorylation and disassociation of some integral junction proteins has been observed [52]. A parasite virulence factor involved in these events has not been identified.

The adherence of *E. histolytica* trophozoites to target epithelial cells is mediated by the Gal-lectin [53]. Lectin activity is inhibited by Gal and GalNAc in such a fashion that the sugars prevent the contact-dependent cell lysis caused by the parasite [54]. In vitro experiments using Chinese hamster ovary (CHO) cell mutants engineered to be deficient in terminal Gal/GalNAc residues revealed that the cells are almost completely resistant to amebic adherence and cytolytic activity [55, 56]. Rapid killing of host target cells occurs within 5-15 minutes after contact with the parasite and the mechanism of action is not clear, but both cell death characteristics of apoptosis and necrosis have been reported [57-59]. Additional factors such as amoebapores have also been linked to the cytopathic effect of the parasite. Amoebapores are pore-forming proteins, which upon contact, are inserted by the parasite into eukaryotic and bacterial cell membranes to form ion channels [60]. This virulence factor causes depolarization of host epithelial monolayers and is directly involved in lysis of target cells [61].

E. histolytica trophozoites disrupt and invade the colonic epithelium after contact, and cysteine proteinases play a key role in this event by killing target cells [62] and degrading extracellular matrix proteins [63]. Secreted and membrane bound cysteine proteinases are involved in trophozoite invasion. The proteinases are responsible for the cytopathic effect, which is the detachment and rounding of epithelial cells in vitro [64] but this event has not been observed in animal models. Following parasite invasion of the epithelium, a massive infiltration and subsequent lysis of neutrophils occurs in the lamina propria, contributing to the inflammation observed during invasive disease and leads to tissue necrosis around the site of invasion [65].

1.5 *E. histolytica* Major Virulence Factors and Their Role in Invasive Disease

The events involved in invasive amebiasis are multifactorial and no single virulence factor alone is responsible for all events of pathogenesis. Three parasite pathogenic factors have been extensively characterized and these include the Gal-lectin, the amoebapores, and the cysteine proteinases. These virulence factors and their suggested functions are summarized in **Table 1.1**. Although other putative parasite molecules may be involved in pathogenesis, and still others may have yet to be defined, these three factors are known to play key roles in invasive amebiasis.

Table 1.1 Major *E. histolytica* Virulence Factors

Virulence Factor	**Suggested Role in Pathogenesis**
Gal/GalNAc-Adherence Lectin	1. Adherence to and colonization of colonic mucus layer. 2. Adherence to target cells. 3. Cytotoxicity 4. Resistance to complement-mediated cytolysis.
Amoebapore proteins A, B, and C	1. Lysis of target cells
Cysteine Proteinases	1. Degradation of mucus barrier? 2. Degradation of extracellular matrix proteins. 3.Degradation of immunoglobulins and complement components 4. Induce colitis

One of the best-characterized virulence factors of *E. histolytica* is the parasite's surface Gal-lectin. The Gal-lectin has been shown to play multiple roles in amebic pathogenesis, which include: colonization of the mucus layer [37], adherence to host cells [66], cytotoxicity [54], resistance to complement-mediated lysis [67], as well as amebic actin polymerization and cell signaling [68, 69]. As stated earlier, colonization of the mucus layer by the parasite is mediated through the Gal-lectin by its ability to bind Gal and GalNAc residues of mucus glycoproteins with high affinity. GalNAc is the preferred sugar substrate over Gal, having a sevenfold higher affinity for the GalNAc monosaccharide. In addition, the lectin has a 1000 times higher affinity for GalNAc containing- over Gal containing-oligosaccharides, and a 100,000

fold higher affinity for polyvalent Gal/GalNAc containing neoglycoproteins than the corresponding monosaccharides [70, 71]. Colonic mucin is highly polyvalent in terminal Gal and GalNAc residues. These findings may explain the parasite's ability to colonize the mucus layer through the high affinity interactions between the parasite lectin and the multivalent nonreducing terminal GalNAc residues. The Gal-lectin is also involved in adherence and contact-dependent cytolysis of human epithelial cells as well as neutrophils, T-lymphocytes, macrophages and erythrocytes [54]. Amebic contact with mammalian cells rapidly results in a 20-fold increase in intracellular calcium and membrane blebbing [72]. The mechanism of target cell death is not entirely understood and has been reported to vary according to cell type. For example, *E. histolytica* causes necrosis to occur in human myeloid cells [59], and apoptosis in murine myeloid cells [73]. Several lines of evidence suggest a non-classical mechanism of apoptotic killing by the parasite. There is evidence that the parasite damages cell membranes through the formation of pores. The plasma membrane then loses its function as a permeability barrier and subsequent cell swelling and lysis occur [59]. This was shown using Jurkat cells in which cellular membranes were compromised prior to DNA degradation [59].

Studies using monoclonal antibodies directed against the heavy subunit of the Gal-lectin, which blocked cytotoxicity but not adherence, directly implicated the Gal-lectin in causing cell death [74]. The mechanism of this action is not known; researchers have theorized that the binding may block conformational changes in the Gal-lectin necessary for cell killing [53]. Antisense inhibition of the 35 kDa light subunit of the Gal-lectin does not strongly affect amebic adherence to target cells, but does inhibit amoeba cytotoxicity and liver abscess formation [75]. These results are not surprising since the carbohydrate recognition domain (CRD) is present on the heavy chain, and suggest that the light subunit has an important role in virulence. The Gal-lectin directly interacts with the parasite's cytoskeleton and upon binding to target cells, the lectin may signal events involved in cytolysis through the stimulation of actin polymerization [68] [76]. Actin polymerization occurs at the site of target cell contact and is involved with parasite motility and target cell interactions including

phagocytosis. The Gal-lectin does not only play a role in recognition and binding of host cells, but it is also involved in parasite survival in the host by inhibiting components of the innate immune response. During migration through host tissue and/or the circulatory system, the parasite is exposed to human complement, but virulent parasites are resistant to complement-mediated lysis [77]. The Gal-lectin binds to C8 and C9 components of complement and blocks the formation of the membrane attack complex on the ameba plasma membrane [78]. The heavy subunit of the Gal-lectin shows a limited homology with CD59, which is a human inhibitor of C5b-9 assembly and may explain the inhibitory activity.

E. histolytica produces three amoebapore proteins which include amoebapore A, B, and C. Isolated amoebapores were found to be cytotoxic towards eukaryotic cells and also have potent antibacterial activity [60]. Their primary function is hypothesized to be for combating the growth of phagocytosed microorganisms inside the digestive compartments [79]. The pore forming proteins are contained within acidic cytoplasmic granules in the parasite and are not constitutively secreted by trophozoites [80, 81]. These proteins share significant homology with NK-lysins found in natural killer cells. Pore forming activity is strongly dependent on pH and amoebapore polymerization occurs only between pH 4 and pH 6, and is triggered by the protonation of a histidine residue [79]. Therefore, the active proteins are likely secreted by the parasite into the intercellular space of the contact zone where a microenvironment, having a low pH could be maintained [82]. Insertion of amoebapores into cellular membranes results in the rapid depolarization of target cells and the formation of ion channels, which allow the passage of water, ions, and other molecules into the cell, leading to lysis [32]. Amoebapore A (AP-A) is the most abundant of the three isoforms produced by the parasite and the proteins are present at a ratio of 35:10:1 for A, B, and C respectively [83] and the molecules have similar structural and functional properties. Antisense inhibition and transcriptional silencing of the AP-A gene has made it possible to define a role for the virulence factor in invasive amebiasis. A 60% reduction in AP-A activity has been achieved using antisense technology in amebic trophozoites [84]. Parasites with reduced amoebapore

activity exhibited a 90% decrease in cytopathic and cytolytic activity towards mammalian cells while retaining only 40% of normal AP-A protein. The parasites also displayed a reduced ability to induce hepatic lesions in hamsters [84]. Additionally, transcriptional silencing of the amoebapore gene confirmed the role of the virulence factor in killing target cells as well as disruption of phagocytosed cells, since silenced parasites were found to be avirulent [85]. These results showed for the first time that the inhibition of an amoebapore directly affects the pathogenicity of the parasite.

Cysteine proteinases are by far one of the most important virulence factors involved in colonic invasion produced by the parasite. This class of amebic protease has been implicated in the pathogenesis of invasive amebiasis due to its involvement in degrading extracellular matrix proteins [64], immunoglobulins [86, 87], anaphylatoxins C3a and C5b [88] and their role in the detachment of tissue culture monolayers [89]. In addition, *E. histolytica* produces anywhere from 10 to 1,000 fold more secreted proteinase activity than non-invasive *E. dispar* [90], and these findings have led to the interest in the role of these enzymes in invasion. See chapter 2 for an extensive review of the structure and function of *E. histolytica* cysteine proteinases.

1.6 Host Response to *E. histolytica* Infection

The host response to *E. histolytica* plays a major role in the pathogenesis of invasive amebiasis. Intestinal epithelial cells are important components of the host's innate and acquired immune system and can produce active cytokines in response to certain stimuli. In addition to parasite virulence factors directly damaging host cells, the parasite initiates an acute inflammatory response by causing the production of proinflammatory cytokines, and chemoattractant factors even in the absence of cellular contact [91, 92]. Coculture studies of the parasite with epithelial and stromal cells have revealed that the parasite induces the production of tumor necrosis factor α (TNF-α), interleukin 1α (IL-1) and interleukin 8 (IL-8). IL-8 is a potent chemoattractant factor of neutrophils and may play a role in the initiation of the inflammatory response even before mucosal invasion [92]. Amebic lesions in animal

models are characterized by an infiltration of polymorphonuclear leukocytes (PMNs), the majority being neutrophils [93]. Interestingly, trophozoites are quite resistant to neutrophil mediated killing while virulent trophozoites effectively kill neutrophils [94]. This massive lysis results in the release of cytotoxic granules which contribute to host tissue damage [95]. Infiltration of neutrophils to local sites where amoeba are invading is a sign of acute inflammation and results in tissue damage. *E. histolytica* cysteine proteinases also possess interleukin-1 beta (IL-1β) converting enzyme activity which may contribute to intestinal inflammation by activating pIL-1β released by damaged cells, contributing to the influx of inflammatory cells into the mucosa [96].

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Chapter 2. *E. histolytica* Cysteine Proteinases

2.1 Structure and Function of *E. histolytica* Cysteine Proteinases

Peptide hydrolases (proteases) are enzymes, which catalyze the cleavage of amide linkages (peptide bonds) and can be categorized based on their substrate specificities or mechanisms of catalysis. For example, five major classes of proteases have been identified based on their mechanism of peptide hydrolysis: serine, cysteine, aspartic, threonine, and metallo-proteases. The most abundant type of enzymes produced by *E. histolytica* are the cysteine proteases, and these have been implicated in the pathogenesis of invasive amebiasis [1]. As a class, cysteine proteases include several mammalian lysosomal cathepsins, caspases, the cytosolic calcium activated proteases (calpains), and the plant proteases papain and actinidin [2]. Cysteine proteases have been identified in many types of parasitic protozoa and helminths including, but not limited to *E. histolytica*, *Leishmania mexicana*, *Plasmodium falciparum*, *Giardia lamblia*, *Trichomonas vaginalis*, *Fasciola hepatica* and *Schistosoma japonicum and S. mansoni* [3, 4]. This class of protease has many functions throughout a parasite's life cycle and is necessary in such events as encystment/excystment, nutrient uptake, protein degradation, growth and development, evasion of the host immune response, as well as invasion of host tissues or cells [5]. In addition, these proteases may play crucial roles in host-parasite interactions and affect the outcome of infection.

Proteinases or endopeptidases are proteases which cleave internal peptide bonds and the term cysteine proteinase is descriptive of the active site mechanism and identifies some characteristics of the substrate specificity for this class of enzyme [6, 7]. *E. histolytica* cysteine proteases have been reported to exhibit endopeptidase activity as determined by protease inhibition and synthetic substrate cleavage studies [1] [8]. In addition, the major cleavage sites on the bovine insulin β-chain have also been identified, supporting the role of these enzymes as being endopeptidases [9]. The majority of parasite cysteine proteases belong to the papain-like family of proteases under the subfamilies of cathepsin-L and cathepsin-B based on structural differences, and at least 9 additional types of cathepsins are found in mammalian cells [10]. *E. histolytica* cysteine proteases (EhCPs) share the

closest structural identity to the cathepsin L-like proteases while exhibiting cathepsin B-like activity by hydrolyzing the synthetic substrate Z-Arg-Arg-pNA [11], indicating that the proteases cannot be classified in the same manner as typical mammalian cysteine proteases [11]. Cathepsins B and L have primary substrate preference at the S_2 subsite and cathepsin B-like proteases preferentially cleave substrates with positively charged amino acids such as lysine or arginine in the P_2 position and have little affinity for cathepsin L substrates with bulky aromatic amino acids in the P_2 position [12] [5]. An example of the interactions between a peptide and the cysteine protease active site pocket is seen in **Fig. 2.1**. These differences can be explained due to the fact that the *E. histolytica* cysteine proteinase (EhCP) active site pocket usually contains a negatively charged aspartic acid in the S_2 subsite, except in the case for EhCP5 where homology modeling predictions indicate a glycine in the S_2 pocket [11]. This difference would account for the cathepsin B-like activity exhibited by the proteinases and may allow for a broader range of substrate specificity for EhCP5, but this has yet to be determined.

In general, mammalian cathepsins are most active in the acidic pH of the lysosome, but become rapidly inactivated at neutral pH and this may be a protective mechanism against escape of these proteases from the lysosomal compartment [6]. Over-expression or aberrant expression of human extracellular cathepsin activity is associated with a variety of diseases including cancer, osteoporosis, inflammation, and rheumatoid arthritis [6, 13, 14]. Cysteine proteases secreted by parasites that invade host tissues and cells are active over a broader range of conditions, and are most active at neutral to slightly alkaline pH [12]. *E. histolytica*, *F. hepatica, Ostertagia ostertagi* and *T. vaginalis* for example secrete cysteine proteases into their environment that are highly active against host proteins [15-18].

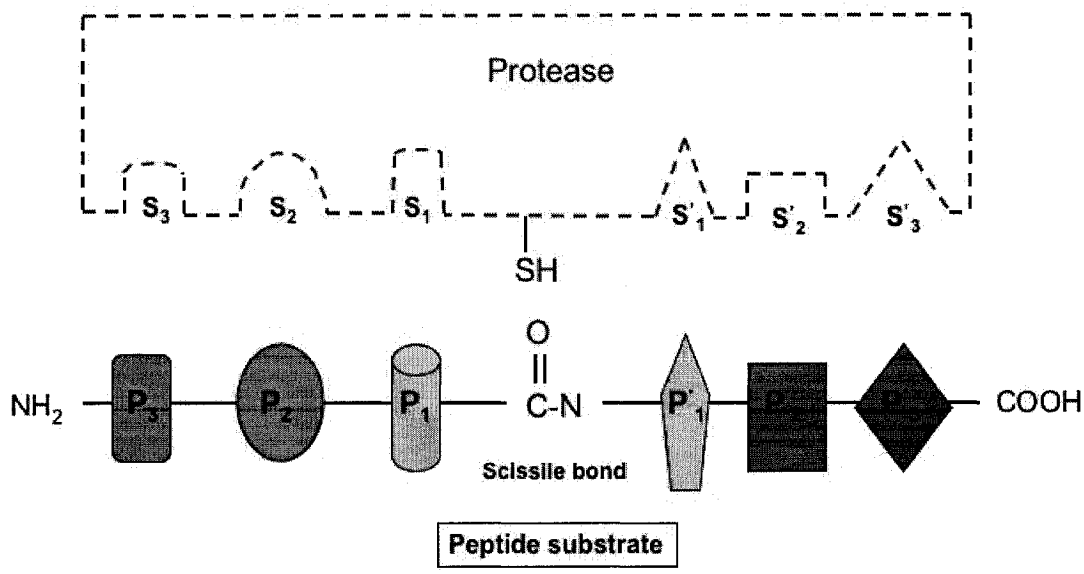

Figure 2.1 Diagram of the peptide substrate interactions within the active site pockets of a cysteine protease. Amino acids from the peptide are designated by a P_n, (n indicating the position of the amino acid from the scissile bond), and the corresponding protease subsites that they interact with are designated by an S_n. The carboxyl side of the peptide and corresponding subsites are given the designation "prime" while amino acids on the amino side are given the non-prime designation. *E. histolytica* cysteine proteinases are most active against substrates with arginine in the P_2 position. Adapted from: [5].

E. histolytica cysteine proteinases (CPs) contain all the key structural features typical of the papain like cysteine proteases including an N-terminal predomain, a propeptide, and a catalytic domain which represents the mature enzyme [19]. **Fig. 2.2** illustrates the basic structure of an *E. histolytica* cysteine proteinase. Amebic predomains range from 12 to 14 amino acids in length and the signal peptide is composed of a basic N-terminal region (n-region), a central hydrophobic region (h-region) and a polar C-terminal region. The predomain region or signal sequence is responsible for translocation of the enzyme to the endoplasmic reticulum. Following ribosomal protein synthesis and processing in the ER, the signal peptide is removed by signal peptidase cleavage between the pre and prodomains, and the cleavage site has been predicted using the method of von Heijne [20]. The propeptide segment of eukaryotic cathepsins has at least three functions [21,

22]: (1) the proregion acts as a structural template, allowing for proper folding of the enzyme, (2) the prodomain functions as a molecular chaperone for the transport of the enzyme through the secretory pathway, and (3) the prodomain acts as a reversible inhibitor of the enzyme and prevents premature activation. *E. histolytica* CP prodomains contain a conserved ERFNIN motif (Glu-X_3-Arg-X_2-(Val/Ile)-Phe-X_2-Asn-X_3-Ile-X_3-Asn) close to amino acid -50 for all six amebic proteinases [23] and this motif is a common structural feature of cathepsin L-like proteases. The mature region of *E. histolytica* cysteine proteinases range in size from 216 to 255 amino acids, are structurally similar to cathepsin L-like proteases, and have an active site that contains a cysteine, histidine, and asparagine residue. The majority of cysteine proteases contain a highly conserved active site sequence, CGS**C**WAFS (active site cysteine underlined), although *E. histolytica* cysteine proteinases contain some amino acid substitutions [11]; for example, EhCP5 contains the sequence CGS**C**YSFA and EhCP1 contains the amino acid sequence CGS**C**YTFG. Finally, a number of cysteine residues involved in disulfide bridge formation conserved in cathepsin L-like enzymes are found in *E. histolytica* cysteine proteinases.

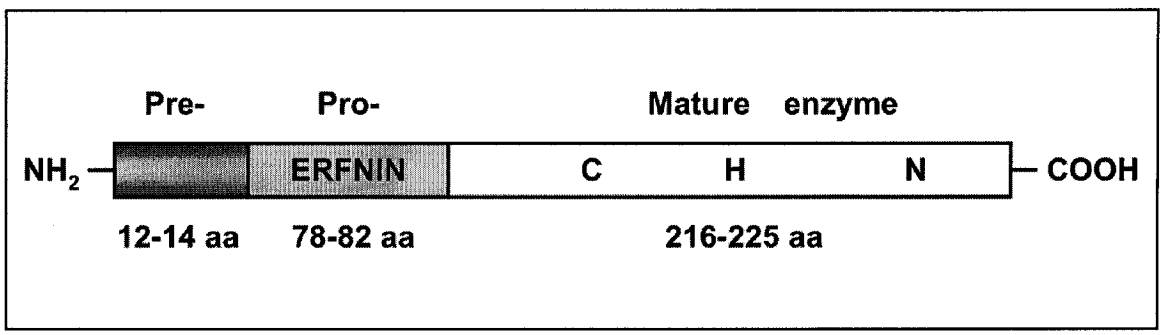

Fig. 2.2 *E. histolytica* cysteine protease structure. Active site residues: (C) cysteine, (H) histidine, (N) asparagine. Adapted from: [19].

2.2 Expression and Regulation of *E. histolytica* Cysteine Proteinase Genes

The *E. histolytica* genome contains 20 cysteine proteinase genes with full-length open reading frames, although only seven are expressed in the parasite during in vitro cultivation [24, 25]. A summary of the major cysteine proteinase genes found in *E. histolytica* and the nonpathogenic commensal *E. dispar* (EdCP) as well as the known substrates for the enzymes (EhCPs) is listed in **Table 2.1.** Functional genes corresponding to *EhCP1* and *EhCP5* are absent in *E. dispar* and have led to investigation into the role of these proteinases as virulence factors. In addition, pathogenic isolates of *E. histolytica* release larger quantities of proteinase than *E. dispar* [15]. The *E. histolytica* cysteine protease genes *EhCP1, EhCP2, EhCP3, EhCP5, EhCP8, EhCP9 and EhCP112* are all expressed at various levels in the parasite [25, 26] with *EhCP1, EhCP2* and *EhCP5* expression being the highest. Expression levels of these genes directly correlate with the amount of cysteine protease activity found in trophozoite lysates. N-terminal sequence analyses of the purified cysteine proteinases from the parasite revealed that ~90% of total cysteine proteinase activity could be attributed to the proteins EhCP1, EhCP2, and EhCP5 [24].

Table 2.1 Cysteine Proteinases of *E. histolytica* and *E. dispar*

Enzyme	Gene Expression		Known Substrates (EhCPs)	References
	E. histolytica	*E. dispar*		
EhCP1* (EdCP1)	+ (S)	-	Laminin, fibronectin, bovine collagen type I, human collagen type IV	[27, 28]
EhCP2* (EdCP2)	+ (S)	+	Human collagen type IV	[8]
EhCP3 (EdCP3)	+ (S)	+	NA	
EhCP4 (EdCP4)	-	-	NA	
EhCP5* (EdCP5)	+ (S)	-	Fibronectin, IgG, C3 and C9 complement components, proIL-18, hemoglobin, fibrinogen	[29-31]
EhCP6 (EdCP6)	-	-	NA	
EhCP8 (EdCP8)	+	+	NA	
EhCP9 (EdCP9)	+	-	NA	
EhCP112 (EdCP112)	+ (S)	-	Collagen type I, fibronectin and hemoglobin	[32]

*Underline indicates highest expression levels of the cysteine proteinases. (S) denotes the enzyme is secreted by the parasite.

CPs are spontaneously secreted by the parasite in the absence of cellular contact [33] and the major secreted proteinases detected in parasite conditioned medium are as follows: EhCP1>EhCP2>EhCP5>EhCP3 (personal communication, Meléndez-López, S.). There is evidence suggesting that the level of CP activity produced by amoebae is directly related to parasite virulence, and is likely due to the action of the proteinases on extracellular matrix proteins such as laminin and fibronectin. The virulent HM-1 (HM-1:IMSS) strain exhibits high levels of cysteine proteinase activity [34, 35]. High levels of CP activity have also been detected in parasites isolated from patients with symptomatic cases of amebic colitis or liver abscess compared to asymptomatic individuals, and antibodies against the proteinases have been detected in infected individuals [15].

Host proteins encountered by the parasite during infection are known to play a role in regulating the expression of amebic cysteine proteinase genes as well as the release of parasite virulence factors. Interactions between amebic trophozoites and human collagen type I (a major component of connective tissue) have been studied using microarray technology [36]. A significant up-regulation of *EhCP1* and *EhCP2* mRNA levels occurs in response to incubation with collagen type I and may give a more accurate prediction of protease activity released within the host [36]. Incubation of the parasites with collagen also leads to parasite activation and release of virulence factors such as collagenase, as well as various other proteases, as detected by gelatin zymogram [37]. This increase in protease production may be needed for the parasite to degrade the extracellular matrix proteins during invasion. Additionally, co-association of the parasite with the bacterium *Escherichia coli* K12 increased parasite CP activity almost four-fold and this increase was attributed to increased expression in *EhCP2* and *EhCP5* transcripts [38].

2.3 Cellular Localization and Description of *E. histolytica* Cysteine Proteinases

EhCP5 is currently one of three known CPs found on the trophozoite surface, in addition, the enzyme is also localized to intracellular compartments [29]. One explanation for this

localization pattern could be that after its release from intracellular granules, the enzyme associates with the trophozoite membrane. Purification of the native protein from the parasite and subsequent homology modeling have revealed some interesting properties of the enzyme [29]. The proteinase has an isoelectric point of 8.12, much higher than the other CPs. The enzyme shows a strong affinity for membranes and the specific activity increases two-fold when associated with liposomes [29]. The nature of the protein-membrane interaction is hydrophobic and may be explained by the hydropathy profile of the protein, revealing a large stretch of hydrophobic amino acids unique to EhCP5. Interestingly, a functional gene encoding for *CP5* is not found in the non-pathogenic species *E. dispar*. A non-coding *CP5* gene in *E. dispar* has been identified but is highly degenerated and contains numerous nucleotide exchanges, insertions and deletions resulting in multiple stop codons in the sequence [39]. It is hypothesized that the *EhCP5* gene started to degenerate in *E. dispar* coincidently when the two organisms began to diverge from a common ancestor.

EhCP112 is another amebic proteinase with some unique features. EhCP112 forms part of the 112 kDa adhesin protein along with a 75 kDa adhesin (Ehadh112) and is localized to the surface of the trophozoite. [26]. Both proteins are encoded by different genes, but may be held together by strong electrostatic forces or covalent bonding. The 112 kDa adhesin complex is translocated during phagocytosis from the plasma membrane to phagocytic vesicles and then is recycled back to the plasma membrane [26]. EhCP112 also contains a putative transmembrane domain near the C-terminus and an RGD sequence (integrin attachment domain) absent in the other cysteine proteases except EhCP5, which may play a role in the contact of the protease with extracellular matrix proteins [26, 40]. The active proteinase may also be secreted by the parasite [32]. EhCP2 is a highly expressed membrane associated CP found on both the cell surface as well as internal membranes in the parasite, and has also been localized to the surface of *E. dispar* (EdCP2) [23]. EhCP3, on the other hand, is located primarily in cytoplasmic granules of *E. histolytica*. After phagocytosis of erythrocytes by the parasite, both EhCP2 and EhCP3 co-localize into phagocytic vesicles [23, 41]. Since many of the cysteine proteinases localize to these vesicles, it is likely that a major role of these enzymes in the parasite is

the digestion of nutrients. These same cysteine proteinases as well as EhCP1 and EhCP5 are also secreted by the parasite, which would imply an additional role for these enzymes in host invasion.

2.4 The Role of Cysteine Proteinases in Immune Evasion

E. histolytica cysteine proteinases have been found to destroy important components of the innate and acquired immune response directed against the parasite. Thus, these enzymes are important virulence factors involved in immune evasion. *E. histolytica* cysteine proteinases activate the alternative pathway of complement by cleaving the components C3 and C5 [42, 43]. Although the parasite is resistant to complement-mediated cell lysis, the molecules released by this cleavage, C3a and C5a, are potent inducers of inflammation. The main role for these anaphylatoxins is to recruit inflammatory cells to active sites of inflammation. C3a is responsible for activating neutrophils, inducing the release of histamine from mast cells, and increasing vascular permeability, as well as inducing the release of IL-1 from macrophages (reviewed in [44]). C5a, the more potent anaphylatoxin, induces macrophage activation [45], chemokinesis and chemotaxis of neutrophils [46], enhances vascular permeability [47], and causes the release of IL-8 [48], IL-1, and IL-6 [49]. These anaphylatoxins have a very short half-life and the biological significance of this activation during infection with the parasite is not known. C5 is more resistant to amebic proteolysis, and cleavage by the parasite requires 10 times more active cysteine proteinase than does C3 cleavage [50]. In addition, C3a and C5a fragments undergo additional cleavage by the proteases and become inactivated, this degradation may be a mechanism used by the parasite to overcome host immunity [50, 51]. Cleavage of C3 also results in the liberation of active C3b-like molecules that participate in the activation of late acting components of complement [52]. EhCP5 has been shown to degrade IL-18 into inactive fragments, which may help to block the host inflammatory response [30], while EhCP2 has been shown to inhibit the biological activity of host chemokines [53].

In addition to degrading complement and host chemokines, the parasite may circumvent the host's immune response by degrading and inactivating immunoglobulins. Parasite

lysate and secreted components have been shown to degrade both serum and secretory IgA [54]. This degradation was inhibited by the specific cysteine protease inhibitor E-64 (trans-epoxysuccinyl-L-leucylamido (4-guanidino) butane). Human and murine IgG are both cleaved by *E. histolytica* cysteine proteinases [55]. Cleaved monoclonal antibody directed against the 29 kDa surface antigen of *E. histolytica* was virtually ineffective at binding trophozoites compared to native antibody. IgG is produced against the parasite in >95% of infected individuals whether symptomatic or asymptomatic but despite this response, the parasite is still able to invade [56]. The cysteine proteinases may limit the effectiveness of the humoral response against the parasite.

2.5 Role of Cysteine Proteinases in the Pathogenesis of Invasive Amebiasis

E. histolytica encounters many obstacles in the host that impede its ability to invade. These obstacles are made up of the innate defenses such as intestinal mucus and antimicrobial peptides as well as soluble host factors such as secretory IgA antibodies. A summary of the innate defenses of the gut is listed in **Table 2.2.** In addition, the parasite must destroy the colonic epithelium by disrupting tight junction proteins that help form a protective barrier from intestinal contents including microorganisms and their secreted products. Once the parasite passes through and disrupts the intestinal epithelium, it meets little resistance from the underlying mucosa and easily migrates through host tissue. The trophozoite may then passively disseminate to the liver via the circulatory system.

Many virulence factors produced by *E. histolytica* are involved in the pathogenesis of invasive amebiasis, but cysteine proteinases are among the most destructive molecules produced by the parasite. Since the trophozoites bind mucin oligosaccharides and the mucus layer is the first innate defense the parasite comes in contact with, one can easily theorize that the parasite disrupts this barrier via proteolysis. Other parasites that come into contact with mucin such as *T. vaginalis* and *F. hepatica* have been shown to degrade mucin, but the specific molecules involved in degradation by these organisms have not been characterized and the degradation may be attributed to protease, as well as glycosidase activity [57, 58]. Since *E. histolytica* secretes glycosidases and proteases into its surroundings, the actions of these enzymes on the mucus layer should not be

discounted [59-61]. An extensive review of the structure and function of gastrointestinal mucin can be found in Chapter 3.

Table 2.2 Innate Defenses of the Gastrointestinal Tract

Molecule	**Defensive role**
Secretory mucins	Mucus gel formation, physical barrier, molecular sieve, reduces shear stress, blocks chemical insults, binding sites for intestinal flora, trap and expel pathogens.
Gastric acid	Antimicrobial [62]
Lysozyme	Antibacterial actions [63]
Antimicrobial peptides (defensins)	Antimicrobial actions [64]
Trefoil factor proteins	Epithelial restitution, epithelial continuity [65]
Mannose-binding lectin	Activation of complement [66]
Epithelium/tight junctions	Mechanical barrier [67]
Galanin-1 receptor/Galanin	Fluid and electrolyte secretion [68]
Toll-like receptors	Innate recognition of pathogens [69]

Adapted from: [70]

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Chapter 3: Production, Structure and Function of Gastrointestinal Mucins[1]

3.1 General Properties of Mucins

The gastrointestinal (GI) epithelium is covered by a thick viscous mucus blanket composed of water, salts, immunoglobulins, secreted proteins and most importantly, mucins. Mucins are high molecular weight glycoproteins, which act as the main structural component, giving rise to the polymeric, viscoelastic and protective properties of the adherent mucous gel. The mucus layer is the most important protective component of the GI tract and all mucosal surfaces due to its ability to maintain epithelial barrier function. Not all mucins are alike and thus, are grouped into two major classes: membrane bound, and secreted mucins. Secreted mucins contribute to the formation of the mucus gel and are produced from specialized epithelial cells found throughout the GI tract, including the salivary glands, stomach, pancreatic and bile ducts, small and large intestines and colon. A summary of the major secreted mucins and their tissue distribution is listed on **Table 3.1.** Membrane-bound mucins are located on epithelial cells throughout the body, but their function is not as well defined. As a class, mucins are extremely large glycoproteins with molecular masses ranging between 0.5-25 x 10^6 kDa. The protein core of a typical mucin molecule contains mucin domains, consisting of tandem repeats rich in the amino acids threonine, proline and/or serine and the hydroxyl residues are heavily substituted with *O*-linked oligosaccharides [1]. The carbohydrate content of mucin may be responsible for more than 90% of its dry weight and the abundant glycosylation of the repeat domains give mucin its characteristic bottle-brush like appearance. Additionally, the densely packed carbohydrates are responsible for protecting the protein core from damage. Many mucins also contain sialic acid and sulfate attached to sugars giving mucin a negative charge under physiological conditions.

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[1] Portions of this chapter were adapted with permission from the following reference: Moncada, D.M. and Chadee, K. Infections of the Gastrointestinal Tract. Ed. Blaser, M.J., Smith, P.D., Ravdin, J.I., Greenberg, H.B., and Guerrant, R.L. 2002. Lippincott Williams & Wilkins. Philadelphia, PA. p. 57-79.

The general properties of mucins, such as protease resistance, high charge density from sialic acid and sulfate residues as well as a large water-holding capacity are attributes of extensive glycosylation. In general, the amino and carboxyl terminal regions of mucin are less glycosylated than the mucin domains and contain a wide range of amino acids, most notably cysteine residues. The cysteines form intramolecular disulfide bonds within the carboxyl and amino terminal regions, as well as intermolecular disulphide bonds between mucin molecules, participating in the polymerization of mucin and enabling formation of the viscoelastic mucus gel.

Table 3.1. Tissue distribution of human secreted mucins

Mucin	Tissue/cell distribution
MUC2	Small and large intestine (goblet cells), salivary gland ducts, inferior turbinates
MUC3*†	Jejunum, ileum, colon, gallbladder, goblet cells and absorptive cells of intestine
MUC5AC	Colon (goblet cells), superficial stomach epithelium, bronchus (mucus glands and ciliated epithelium), inferior turbinates endocervical epithelium
MUC5B	Submandibular glanda, salivary glands, gall bladder (billiary epithelial cells), bronchus (mucus and serous glands), colon (goblet cells), endocervical epithelium, inferior turbinates (submucosal glands).
MUC 6	Small and large intestine (goblet cells), gall bladder epithelium, stomach (mucous neck cells;antral mucous cells), seminal vesicle, pancreas (centroacinar cells and ducts), endocervical epithelium, endometrial epithelium, biliary epithelial cells.
MUC7	Salivary glands (mucous cells), bronchial airways (submucosal glands), inferior turbinates (submucosal glands)

*Reports of both membrane and secreted forms produced by alternative splicing [2]. †Evidence for two genes encoding MUC3 termed *MUC3A* and *MUC3B* [3].

The surface of the GI epithelium is continually exposed to numerous macromolecules and microorganisms including, chemical irritants, digested foods, toxins, resident bacteria, intestinal pathogens and their products. The mesh-like structure of the mucin gel impedes the diffusion of offending macromolecules through it. The delicate single-cell-thick epithelium lining the intestinal tract would be susceptible to injury from acids and luminal contents if it were not for the non-specific protection provided by the mucus blanket [4]. Not only does the mucus gel protect against chemical insults, but it also provides a physical barrier against enteric pathogens by containing binding sites for resident flora while maintaining high concentrations of secretory IgA [5].

Mucin plays a major role in infections of the GI tract by providing initial attachment sites for mucosal pathogens allowing colonization and establishment of the organisms in the mucus layer. Many invading pathogens secrete enzymes and putative mucin secretagogues that weaken the mucus barrier, facilitating access to the epithelial surface. Alternatively, mucins may prevent invading microbes from gaining access to the mucosa by physically trapping and aiding in expulsion of the organisms. The ability of goblet cells to hypersecrete mucin, along with release of fluid from enterocytes may aid in the rapid expulsion of pathogens. The fate of mucin-bound organisms is dependent upon their ability to successfully colonize the intestinal tract. Most microorganisms are not able to colonize the mucin barrier and are sloughed away with peristaltic movements and expelled during defecation. In addition, nonpathogenic intestinal flora residing in the GI tract play an important role in preventing colonization of pathogens by occupying available microbial attachment sites. Alterations in the mucin barrier are most likely a contributing factor in the pathogenesis of many infections and disease states. The mechanisms leading to alterations in mucus composition in relation to mucin-pathogen interactions are still poorly understood.

3.2 Unregulated/Baseline Secretion

Under normal physiological conditions, goblet cells continually synthesize and secrete mucins that are not stored in the apical granule mass to replenish the mucus blanket covering the epithelium. This continual secretion is necessary to maintain the thickness

of the mucus gel, which is constantly exposed to acids and irritants in addition to being sloughed away via peristaltic movements [6]. The release of newly formed mucin granules during unregulated (baseline) secretion in not a receptor-mediated event. Mucosal explants of human and rat colon have been shown to continually incorporate radio-labeled mucin precursors into mucus glycoproteins [7]. Following synthesis, labeled mucins are then packaged into granules, transported to the cell surface and secreted into the lumen. Stored mucin granules are thought to play a role in unregulated secretion since there is evidence for incorporation of newly formed mucins into storage granules [8]. Little is known about the mechanism of unregulated secretion; however, it is dependent upon continuous transport of granules from the golgi vesicles to the cell surface and movement of granules within the cell is a microtubule-dependent event [9]. Baseline granule turnover is a result of the movement of newly synthesized mucin granules along the periphery of the theca to the apical plasma membrane for exocytosis. Microtubules (MT) play a major role in baseline secretion by maintaining the orderly movement of mucin granules from the golgi to the apex of the cell, through interactions with golgi elements in the supranuclear region and mucin granules located in the peripheral apical granule mass. Monensin, which disrupts golgi function was found to almost completely inhibit baseline secretion in the colonic adenocarcinoma cell line LS180 and agents such as nocadazole which inhibit microtubule assembly also inhibit secretion [9]. These studies suggest that baseline secretion is a result of newly synthesized mucin being transported from the golgi to the cell surface with the assistance of MTs.

3.3 Mucin Secretion in Response to Gastrointestinal Pathogens

Mucin secretion is enhanced in response to offending microorganisms. Goblet cells are responsible for maintaining the mucus blanket and are known to respond to various luminal pathogens and their products through an increase in mucus secretion [10-12]. This rapid secretion is thought to bestow an important mechanism of protection by trapping and flushing out intestinal pathogens. Bacteria and their toxins and endoproducts are known to have a potent secretagogue effect on goblet cells; for example, in addition to causing severe diarrhea in humans, *Vibrio cholerae* enterotoxin

was shown to markedly increase mucin secretion in the rat small intestine, colon and HT-29 cells [13]. The bacillus *Yersinia enterocolitica* is linked to an increase in mucin secretion in rabbit distal small intestine and the proximal colon [14]. Perhaps the best example illustrating mucin-pathogen interactions may be observed during infection with the enteric protozoan parasite *E. histolytica*. The parasite colonizes the colon by binding to Gal and GalNAc residues of colonic mucin, and is known to evoke the release of mucins through protein kinase C activation in LS 174T cells [15]. Not only do goblet cells respond to *E. histolytica* by secreting mucins, but the parasite also stimulates the release of both pre-formed and newly synthesized mucins [16], as well as both neutral and acidic mucin species [10]. This rapid secretion does not result in expulsion of the parasite but may result in depletion of the mucus blanket at a rate that exceeds regeneration, allowing the parasite to gain access to the colonic epithelium. This may be a novel strategy used by an intestinal pathogen to gain access to the underlying epithelial cells. Infection with *H. pylori* is also associated with depletion of the gastric mucus barrier, but instead of causing mucin hypersecretion, the organism inhibits mucin biosynthesis [17]. Ironically, pathogens can cause a depletion of the mucus blanket, either by inducing hypersecretion of stored mucin pools or by decreasing mucin biosynthesis.

3.4 Structure of Gastrointestinal Mucins

Peptide core

The peptide core of mucins has been reported to range in size from approximately 1,500 amino acids to more than 5,000 amino acids in composition for the MUC2 apoprotein. The polypeptide backbone provides numerous sites for the addition of *O*-linked oligosaccharides through an abundance of serine and threonine residues. The amino acid composition of mucin contributes to only ~10 to 20% of the dry weight of a typical secretory mucin molecule and the remainder of the protein is composed of *O*-linked and *N*-linked oligosaccharides and a small percentage of sulfate residues [17]. There are currently at least four secreted mucins found covering mucosal surfaces in humans; these are MUC2, MUC5AC, MUC5B, and MUC6 and the genes encodong these mucins are clustered on chromosome 11p15.5 [18]. Advancements in the discovery and cloning of mucins have revealed that there are several characteristics common among these

molecules. Intestinal apomucins contain common structural domains including a series of tandem repeats rich in threonine, proline and often serine residues. These mucin domains often contain numerous amino acid repeats and provide a scaffold for abundant *O*-glycosylation. These highly glycosylated regions take on a rod-like, extended conformation due to the presence of numerous oligosaccharides and proline residues, are devoid of any secondary structure, and are less flexible than the poorly glycosylated regions. In addition, the amino and carboxyl terminal ends of secreted GI mucins are rich in cysteine residues, which play a role in polymerization and formation of the mucus gel. The cysteine-rich flanking regions in secretory GI mucins contain D-domains sharing similarity to the D-domains in the blood coagulation factor, von Willebrand Factor (VWF) [19].

One of the most well characterized and studied GI mucin is MUC2. MUC2 is the major secretory mucin produced by goblet cells of the small and large intestines, and is the predominant gel-forming mucin constituting the mucus layer overlying these organs. A diagram representing the structure of a MUC2 monomer is shown in **Fig. 3.1.**

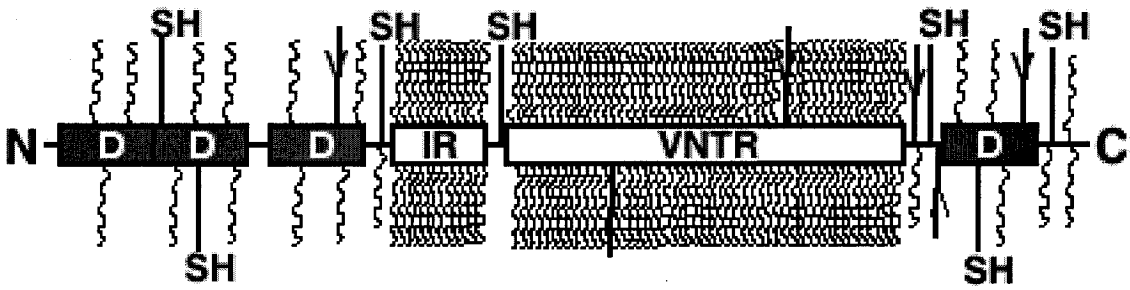

Figure 3.1. Hypothetical model of a MUC2 mucin monomer. The heavily glycosylated mucin domains termed the irregular repeat (IR) region and the variable number tandem repeat region (VNTR) give mucin its bottle-brush like appearance and protect the protein core from damage. The less glycosylated D–domains are rich in cysteine residues forming intramolecular disulphide bonds giving the carboxyl and amino terminus a globular protein appearance. IR, irregular repeat, VNTR, Variable number tandem repeat,

D, von Willebrand factor like domains, and lines indicate area of glycosylation. Adapted from: [20, 21].

At least 90% of *MUC2* alleles encode a protein consisting of more than 5000 amino acid residues [1, 22, 23]. The MUC2 apoprotein contains two mucin domains termed the variable number tandem repeat (VNTR) composed mainly of the amino acids threonine and proline, and the irregular repeat region (IR) rich in serine, threonine and proline residues. The VNTR is composed of a series of 23 amino acid repeats and provides several potential sites for *O*-linked glycosylation. The IR is composed of a 347-amino acid domain, also providing several *O*-linked glycosylation sites, contributing to the resistant nature of these domains to chemical and proteolytic damage [1]. The N-terminus of MUC2 flanking the IR region contains three cysteine-rich D-domains and the carboxyl terminal end of MUC2 flanking the VNTR possesses one cysteine rich D-domain. These domains share high sequence similarity to the D-domains of VWF and are named D1-D4 in order from the N- to C-terminus of the molecule. These regions are involved in the formation of MUC2 monomers into polymers, characteristic of large secretory mucins. Potential *N*-linked glycosylation sites are also dispersed throughout the molecule, although much less prominent than *O*-linked oligosaccharides.

Amino Terminal End

There are several cysteine rich domains found in human secreted mucins. These domains are often at the terminal regions of mucin molecules and they share a high sequence similarity in the position of the cysteines when compared to the VWF. These regions are also named similarly to the D-domains of VWF termed D1, D2 and D3. In addition, a partial D' domain is located between the D2 and D3 in all VWF-like secreted mucins as well as in VWF [24]. Secretory mucins often contain three N-terminal flanking VWF-like domains as depicted in **Figure 3.1** for MUC2. In addition, these regions may have as many as 30 cysteine residues participating in the assembly of mucins into multimers by the formation of disulfide bonds. All cysteine residues in VWF are thought to participate in inter- or intra-molecular disulfide bond formation [24]. The N-terminal of MUC2 and other mucins contain sites for potential *N*-linked glycosylation, which are essential for the

proper processing and transport of mucin within the secretory pathway [25]. The N-terminus of MUC2 is assembled into trimers, held together by disulfide bonds between monomers of similar termini [26]. Trefoil-like domains have been identified within this region and allow the polymer to stay intact even after digestion with trypsin, due to disulfide bonds holding the proteins together [26]. This unique structure may allow the mucin polymer to maintain its structure even under the constant assault of intestinal proteases.

Carboxyl Terminal End

The C-terminal regions of most secreted mucins contain a single D-domain rich in cysteine residues aligning similarly with those of the VWF domains. This suggests an important function for this region. Most secreted mucins contain one C-terminal D-domain, termed D4, in keeping with the naming in order from N- to C- terminus. The carboxyl terminal D-domain of mucin takes on the structure of a globular protein and is highly homologous to the cysteine knot motif family of proteins [25] [27]. Currently it is believed that all available thiols participate in either inter- or intra-molecular disulfide bond formation in a similar fashion to those in VWF [24]. Sites for *N*-glycosylation may also be present in this region, but there are little or no potential sites for *O*-linked glycosylation. Dimerization and subsequent polymerization of mucin is thought to occur in a similar fashion as VWF, through the C-terminal by a "tail to tail" assembly. Reducing agents disrupt the polymeric structure by breaking the intermolecular disulfide bonds between mucin molecules, although reduction insensitive bonds have been identified [1]. MUC2 has a unique C-terminal region. Exposure of MUC2 mucin to reducing agents often results in the release of a 118 kDa peptide from the C-terminus that is easily visualized when separated by SDS-polyacrylamide gel electrophoresis. Previously thought to be a link peptide, further analysis revealed the sequence to be part of the C-terminal peptide core. Cleavage of MUC2 mucin by an unknown mechanism was thought to be responsible for the release of this peptide under normal conditions, and the fragment could remain attached via disulfide bonds [27] [28]. The liberation of a 118 kDa peptide and other mucin glycopeptide fragments often occurs during mucin purification. More recent studies have revealed that the MUC2 C-terminus does undergo

an autocatalytic cleavage between an Asp-Pro peptide bond, in a low pH environment (below pH 6.0) and is only released upon reduction [29]. This cleavage is likely responsible for liberation of the "link peptide" and is not prevented by protease inhibitors [29]. The cleavage is also theorized to be a natural phenomenon that may take place in the late secretory pathway where the pH is below 6.0 and is dependent upon the length of exposure of the molecules to acidic conditions. In addition, the reaction may produce a reactive C-terminus that could link to other components. Purification of mucin in the presence of guanidinium chloride is known to be an effective way to significantly prevent degradation during the purification and storage of mucin.

Tandem Repeats

Epithelial mucins are characterized by their tandem repeat (TR) domains (**Fig. 3.1**), which can comprise more than 50% of the apomucin. The TRs provide sites for *O*-glycosylation, however the content and order of threonine, proline and/or serine residues influences the actual extent of oligosaccharide density. MUC1 contains less serine and threonine residues when compared to MUC5AC and as a consequence, has less glycosylation sites available; in contrast, MUC2 TRs contain a greater concentration of threonine residues and therefore are more densely glycosylated. The 23 amino acid TR of MUC2 (PTTTPITTTTTVTPTPTYTGTQT) contains 14 threonine residues, which have been reported to be glycosylated up to 78% in LS174T cells [30]. The extent of glycosylation can have a dramatic effect on the physical properties of mucin and the TR can influence the rigidity and gel forming properties of mucin depending on the availability of glycosylation sites. The sequence of the repeat may vary between different mucins depending on the function of the molecule, but repeats from all mucins are known to contain threonine, proline and/or serine residues. The presence of proline residues within these repeats appears to be important in determining the specificity of the polypeptide *N*-acetyl-galactosaminyltransferases (GalNAc transferases) catalyzing *O*-linked oligosaccharide addition. The six or seven flanking amino acids around the target hydroxy amino acid influence its acceptor function, especially proline at the position +3, favoring glycosylation of threonine [31-33].

Polymorpisms

Theoretically, mucins are the most polymorphic of all the biological macromolecules produced by eukaryotic organisms. They are even more polymorphic than immunoglobulin and T-cell receptors due to the abundant potential sites for *O*-linked glycosylation and the numerous possibilities for unique and extended oligosaccharide chain combinations. Mucin genes exhibit genetic polymorphism and there are allelic variations between individuals. As a consequence, there are different protein isoforms. Polymorphism in the VNTR are a common feature of most mucins and there is a high level of genetically determined polymorphism due to variations in the number of copies of these tandem repeated sequences (VNTR) [34, 35]. Interestingly, the secretory mucins MUC2 and MUC6 show an incredible degree of polymorphism in the VNTR region, with up to a two-fold difference in length of the coding sequence and subsequently an increase in protein size [35]. Variations in the length of secreted mucin molecules may have an influence on the properties of the mucus gel.

3.5 Carbohydrate Structure

N-linked Oligosaccharides

N-linked oligosaccharides are present in most mucins, although they comprise only a small percent of the total carbohydrates. These carbohydrates are mostly confined to the N- and C-terminal regions of the mucin molecule. Experiments using LS 174T cells treated with tunicamycin, a potent inhibitor of *N*-linked glycosylation, revealed the importance of *N*-glycosylation in the synthesis of the MUC2 apomucin [36] [25]. Despite the lack of *N*-glycans, MUC2 monomers are able to form dimers in the ER but the rate of dimerization is delayed. There is a high probability that *N*-glycosylation may be important in mediating proper folding and disulfide bond formation in the MUC2 apomucin. In addition, the successful transfer of mucin dimers to the golgi for further processing was also found to be dependent on *N*-linked glycosylation of mucin in the ER [25]. In MUC2, there are several consensus sequences for potential *N*-glycosylation sites (asn-X-ser/thr) and some of these sites are known to be occupied, although it is unknown if all available sites are glycosylated. Few compositional or structural details concerning the *N*-linked branches in mucin are known, although at least some of them in rat and

human intestinal mucins must have exposed oligomannosyl residues, based on their recognition of *E. coli* type 1 (mannose sensitive) pili [37].

O-Linked Oligosaccharides

The incorporation of *O*-linked oligosaccharides into mucins occurs following *N*-glycosylation and disulfide linked dimer formation [27]. The functions of *O*-glycans are diverse and include maintaining protein conformation, control of active epitopes and antigenicity, in addition to acting as binding sites for microbes. *O*-glycans are attached to the apomucin peptide TRs via an *O*-glycosidic linkage between the first carbon of GalNAc and the hydroxyl oxygen of threonine or serine. It is possible that these linkages are further stabilized, and the chains oriented with respect to the peptide, by a hydrogen bond between the amide group of GalNAc and the carbonyl oxygen of the threonine or serine [38]. The addition of GalNAc serves to "stiffen" the TR domain of the peptide core in an extended conformation [39]. GalNAc residues are important in maintaining a highly extended random coil configuration of mucin, and removal of the mucin oligosaccharide branches results in the denaturation and collapse of the molecule [39, 40]. These mucin-bound carbohydrates also have a large water holding capacity, which allows for hydration of the mucin molecule. Although *O*-glycans are tightly packed side by side in the central TRs (**Fig. 3.1**), not all serine and threonine residues are necessarily glycosylated and the carbohydrate chains may exist as clusters possibly exposing regions of the TR to damage [40]. This may not be the case for MUC2, since digestion of the MUC2 monomer by trypsin and analysis of the degradation products revealed that the IR and VNTR domains remain intact and correspond to the appropriate predicted molecular weights of the domains [1]. A consensus sequence for the addition of GalNAc has not yet been found, although predicted algorithms do exist. The presence of adjacent proline residues at the –1 and +3 positions has been associated with *O*-glycosylation and the presence of charged residues in these positions is not favored. Proline residues are responsible for breaking helix formation and instead may promote the formation of β sheets, and *O*-glycosylation is thought to occur at these predicted β turns. Alanine, serine and threonine are often found adjacent to *O*-glycosylated residues and apparently charge distribution is more of a major factor vs. actual charge [41]. The sequence of the MUC2

tandem repeat contains consecutive and alternating threonine residues. Studies conducted to predict glycosylation patterns in MUC2 mucin have been performed using synthetic peptides representing various sequences of the VNTR of MUC2. These peptides were incubated with LS 174T cell microsome fractions to determine the incorporation of GalNAc into threonine residues, and it was revealed that maximum incorporation occurred in peptides containing consecutive threonine residues [42].

O-glycans contain GalNAc, GlcNAc, fucose, galactose, and sialic acid. MUC2 contains 21 separate oligosaccharide groups including 10 acidic and 11 neutral structures ranging in chain length size from two to 12 sugars. Many of these may represent minor variations of a basic biantennary structure [43]. Several hundreds of different *O*-glycan structures have been described in mucins, made possible due to variations in linkage (α or β) and degree of branching, which amplify their potential to generate numerous recognition sites for lectins, and viral, bacterial, or parasite adhesins. The carbohydrate structures of mucins are heterogeneous and vary in different regions of the intestine, and even among different mucus cells of the same organ, such as the deep superficial mucus cells of the stomach [44] or the crypt and surface goblet cells of the colon [45] .

The initiating event of *O*-glycosylation is the addition of the monosaccharide GalNAc (from UDP-GalNAc) to serine or threonine residues. *O*-glycan synthesis is simpler than *N*-glycan synthesis as it does not utilize a lipid-linked oligosaccharide precursor for transfer of the oligosaccharide to the apomucin. This addition is catalyzed by a polypeptide GalNAc transferase (GalNAcT). There are several GalNAcTs expressed in various tissues. GalNAcT-1 expression seems to be widespread and abundant in human and other vertebrate cells and there are at least eight polypeptide GalNAcT genes, which are generally expressed in specific tissues and cell types. Specificity for glycosylation by GalNAcTs is regulated both by the enzyme source (organs) and by the apomucin sequence [46]. Typical core structures of mucin *O*-glycans consist of six different arrangements of Gal and GlcNAc bound to GalNAc (**Figure 3.2**). Most *O*-glycans contain the core 1 subtype structure formed by the addition of galactose in a β1-3 linkage to the GalNAc. The first four cores are common, particulary core 2 and 3 in intestinal

mucins. The glycosyltransferase responsible for the Core 1 subtype structure is known as core 1 β1-3 galactosyltransferase (Core 1 GalT). Core 2 GlcNAcT for the formation of the Core 2 subtype, Core 3 GlcNAcT for the Core 3 subtype and Core 4 GlcNAcT for the Core 4 subtype [47, 48]. The core structures are substrates for transferases, which add sugars and elongate the oligosaccharide chains. The production of core 2 *O*-glycans requires the Core 1 as substrate and production of Core 3 *O*-glycans actually inhibits the ability of Core 2 GlcNAcT to act. In addition, the Core 2 structure can become elongated into either a mono- or biantennary form with the presence of multiple lactosamine (Galβ1-4GlcNAc) units, which become terminated by the addition of sialic acid or fucose. The Core 3 *O*-glycans can be the building block for the formation of biantennary *O*-glycans by acting as a substrate for Core 4 GlcNAc activity. Very few tissues besides the GI tract show high Core 3 and 4 GlcNAcT activities. As oligosaccharide elongation proceeds, the more proximal core structures become "masked" making them inaccessible to lectins, adhesins, or antibodies specific for them.

The final step in oligosaccharide synthesis is the transfer of sugars (fucose, galactose, GalNAc, and sialic acid) from their nucleotide sugar donors to terminal galactose residues of the backbones, completing oligosaccharide synthesis. The peripheral sugars bond via α-glycosidic linkages, preventing further elongation, and give rise to the well-known ABH and Lewis blood group specific mucins. The A-, B-, or H-specific sugars can serve as nutrients for some colonic commensals [49]. To some extent, the blood groups determine which species or strains inhabit the human colon. Alterations in peripheral sugars also occur in mucins during immune responses to parasitic infections [50-52].

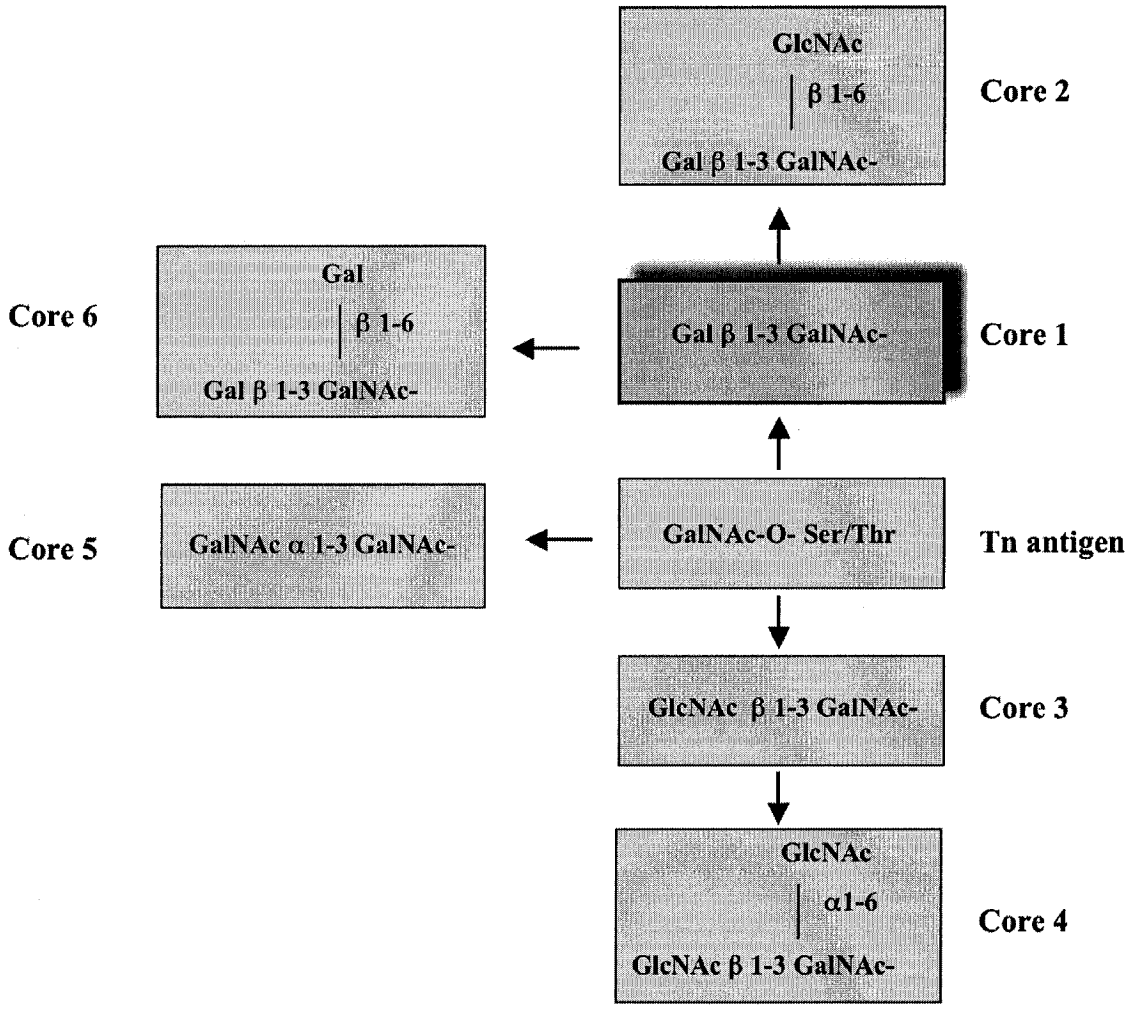

Figure 3.2. *O*-glycan core structures. The mucin cores are synthesized from the precursor Tn antigen by addition of Gal and/or GlcNAc. Cores 1 to 4 are most common in normal mucins.

Sulfation

Some of the peripheral or backbone residues (chiefly Gal or GlcNAc) of mucin oligosaccharides acquire sulfate at the level of the golgi membranes during biosynthesis [53]. The longest or most branched chains are likely to carry the most sulfate [54]. Most goblet cells contain some sulfated mucin molecules and sulfation increases from the proximal to distal segments of the intestine and is highest in colonic areas that harbor

high populations of fecal bacteria and may make the mucin more resistant to degradation [55].

3.6 Mucin Polymers

The interactions between mucins at the intermolecular and intramolecular levels give rise to the polymeric and eventually the viscoelastic properties of the mucus gel. To achieve this, mucin monomers must link together to form polymers. The formation of disulfide bonds between MUC2 mucin monomers is a crucial step in the assembly of mucins into multimers. The assembly of gel-forming mucin polymers is believed to occur in a similar fashion to that of VWF due to the high degree of sequence similarities in the positions of the cysteines in the carboxyl and amino termini. The initial dimerization of MUC2 has been shown to occur in the ER [25] through the formation of disulfide bonds between the carboxyl terminal regions of the mucin peptide. Dimerization of mucin monomers occurs directly after translation of the apoprotein in LS 174T cells. Transfer of the mucin monomers and dimers to the golgi apparatus is an *N*-glycosylation dependent event. Once translocated to the golgi, mucin dimers become *O*-glycosylated. Multimerization of the mucin dimers occurs through interchain disulfide bonds formed between the amino terminal D-domains of disulfide linked dimers, forming very high molecular weight multimers [24, 25, 56]. A hypothetical model of a MUC2 polymer is seen in **Fig. 3.3.**

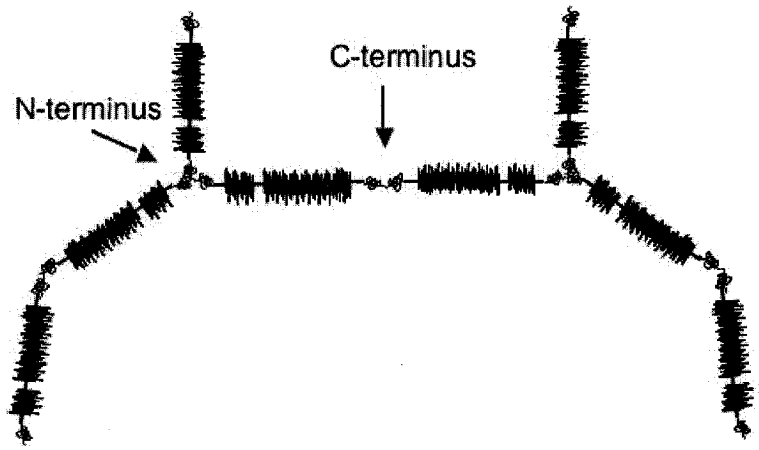

Figure 3.3. Hypothetical model of a MUC2 mucin polymer. The sulfhydryl bonds at the

carboxyl- and amino-terminal ends link the MUC2 monomers. The heavily glycosylated IR and VNTR are depicted as bottle-brush like regions.

Once mucin polymers are assembled and fully glycosylated, they normally assume semiflexible "kinky" configurations which, when extended by shearing stress and examined by electron microscopy, are seen to be extremely heterogeneous in length [57]. Respiratory mucins are well studied and shown to range from 0.2 to more than 10 μm in length. After reduction they decrease in size to 200 to 600 nm [58].

3.7 Gel Formation

Within goblet cell granules, mucin polymers are physically constrained in a highly condensed form, which excludes water. Packing is enhanced by calcium ion neutralization of the fixed negative charges of sulfate and sialic acid. Upon secretion of the mature mucin granules, polymers uncoil, Ca^{+} diffuses outward, and the mucin becomes rapidly hydrated and its volume greatly increases [59]. Mucin molecules in solution aggregate via H-bonding, electrostatic and hydrophobic interactions and Van der Waals forces [60]. Gel formation occurs as the number of cross-links between mucin molecules increases and the long polymer chains intertangle. The sol-gel-phase transformation from the solution of linear or branched molecules to a highly viscous and elastic gel occurs when mucin reaches a concentration of 30 to 50 mg/ml. Cross-linkage of the gel structure provides considerable resistance to flow and may explain why mucins are not completely cleared from the mucosal surface after a single fluid "flush".

Mucin forms a gel that is adherent to the intestinal epithelium, and is composed of two layers: a loosely adherent layer, which is removable, and a layer, which is firmly attached to the mucosa. The mucus gel flows slowly over the mucosal surface forming a blanket, which follows the surface of the mucosa. The viscoelastic properties of mucus can withstand the large shear forces found in the digestive tract as well as the movement of particles and macromolecules over the epithelial surface. If subjected to a strong shear stress, a mucin gel may rupture, but if left undisturbed, it will reanneal due to its instrinsic elasticity. Thus, viscosity and elasticity are important properties for the continuity and

stability of the mucus blanket covering the GI mucosal surface. The unstirred mucus layer provides a stable microenvironment at the mucosal surface, and in the colon, the mucus layer provides an essential environment for microflora [61]. The thickness of the mucus gel ranges from 50 to 450 μm (average 180 μm) in the human stomach and is thinner and possibly discontinuous in the small intestine. Studies measuring the thickness of intestinal mucus in the rat have shown that the mucus layer is thickest in the colon (830 μm) and thinnest in the jejunum (~123 μm) [62]. The mucus layer is decreased during starvation [63] and thickens in bacterial overgrowth [64].

3.8 Susceptibility of Mucins to Damage

Both the peptide core and the oligosaccharides of GI mucins are fragmented and eventually completely degraded by enzymes liberated into the lumen from normal host tissue and colonic bacteria (**Fig. 3.4**). Degradation of mucin is a natural physiological process that plays a role in regulating the thickness of the GI mucus blanket. However, many pathogens and host cells elaborate enzymes that are mucolytic. An excess of these enzymes can alter the dynamic equilibrium between mucin production and degradation rates that can cause a structural weakening of the protective mucus gel layer. Some of the sites on mucin molecules recognized to be particularly susceptible to damage, are discussed below and are highlighted schematically in **Fig. 3.4**.

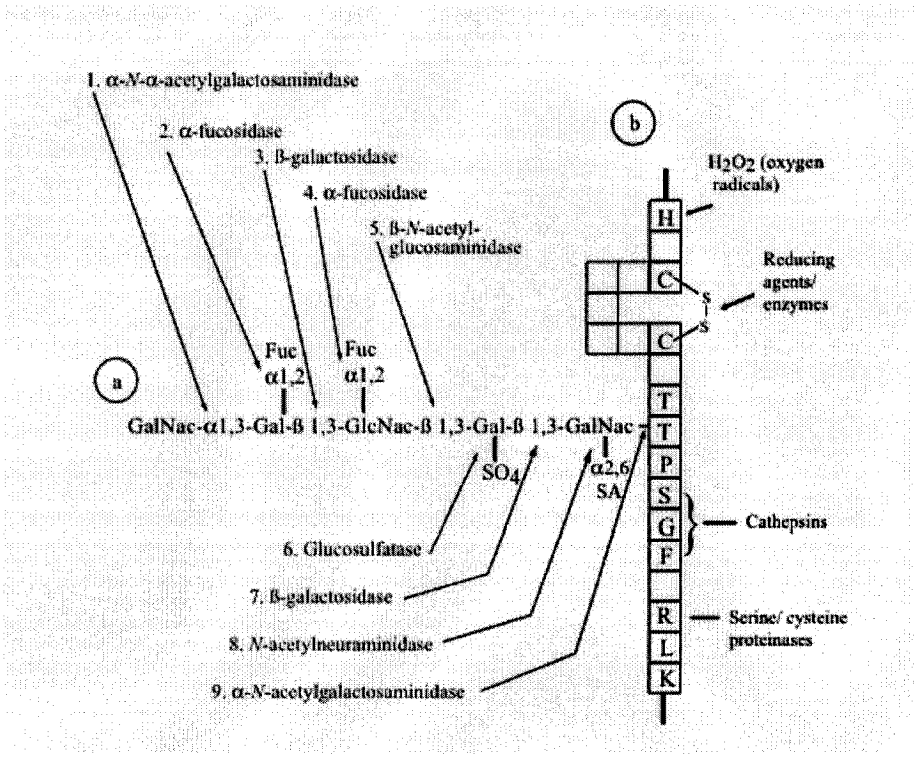

Figure 3.4. Degradation of intestinal mucin. (a)**:** A hypothetical mucin oligosaccharide to show the sequential action of bacterial exoglycosidases and glucosulfatase. (b): A portion of the polypeptide core to show amino acids (squares) vulnerable to chemical and enzymatic rupture. H, histidine; T, threonine; S, serine; P, proline; R, arginine; F, phenylalanine; K, lysine; G, glycine; C, cysteine. Many serine and cysteine proteinases of bacterial and host epithelial cells are mucolytic.

Proteolysis

Solubilization of the intestinal mucus gel occurs throughout the gut by degradation of the polymer into soluble degraded mucin units. The non-glycosylated regions of mucins are theorized to be susceptible to proteolytic cleavage by proteinases. A large portion of the proteolysis of GI mucin takes place in the colon, where there is a high content of intestinal microflora. In addition, mucin in the upper GI tract is constantly degraded by host proteinases. Analysis of the amino acid sequence of MUC2 has revealed that the N- and C- terminal regions contain many arginine, lysine and valine residues as well as a small number of aromatic residues such as phenylalanine, tyrosine, and tryptophan.

Therefore, these domains may be highly susceptible to serine proteinases produced by the host as well as serine and cysteine proteinases produced by GI pathogens. In vivo, some protection of mucins against lumenal proteases may be afforded by weak interactions of mucins with nonmucin components such as proteins, constituents of bile, anionic proteoglycans, lipids, and products of sloughed cells [65, 66]. It is also likely that the normally folded state of the mucin peptide near the cysteine-rich termini "buries" some protease-sensitive regions, which only become vulnerable after disulfide bonds are ruptured [26, 57].

It is hypothesized that once the polymeric structure has been lost, the mucus blanket is less effective at preventing invasion of the intestines. *V. cholerae* secrete a potent zinc-dependent metalloproteinase that lowers mucin viscosity and facilitates penetration of the enterotoxin to its GM1 ganglioside receptor on cell membranes [67]. There is evidence that *H. pylori* secretes a protease that increases the movement of the organism through the mucus gel to the gastric cells [68]. The mucus barrier may also be damaged in inflammatory bowel disease by fecal proteinases, which may be increased by alterations in the normal resident bacterial populations [69]. Additionally, it is likely that GI inflammation contributes to mucin damage by the action of neutrophil proteases [70], mast cell proteases [71], and proteases from other inflammatory cells.

Cysteine residues form disulfide bonds that stabilize the tertiary structure of mucins, and reducing agents are widely recognized to be capable of destroying mucin polymers and collapsing mucin gels. Although specific bacterial reductases for these bonds have not been identified, colonic flora and host cells produce reductases and glutathione, both of which are believed to participate in mucin depolymerization. Thiols in secretions can also activate cathepsins, which can damage the mucin peptide.

Glycosidases

Mucin oligosaccharides are degraded in a stepwise manner by the sequential removal of carbohydrates in a direction from the periphery to the internal core nearest the peptide linkage as shown schematically in **Fig. 3.4**. Specific subpopulations of normal colonic

flora elaborate the required exoglycosidases [69, 72]. The importance of these enzymes as virulence factors, however, is largely speculative. Antibiotics decrease mucin degradation and alter mucin composition to resemble that of mucins found in the germ free state [73]. Proteinases, as well as glycosidases have been shown to play a role in host-parasite interactions, in particular, mucus penetration. There is evidence to support that the protozoan parasites *E. histolytica* and *G. lamblia* produce a β-*N*-acetyglucosaminidase that may aid in the penetration of the protective mucin layer [74, 75]. For organisms such as *T. vaginalis*, motility alone is not sufficient for these pathogens to migrate through the mucus layer [76]. Therefore, the concerted action of proteinases and glycosidases may be necessary for some pathogens to invade the mucosa. *F. hepatica* also produces a range of glycosidases capable of degrading mucin which may facilitate parasite invasion and tissue migration [77, 78] As mucin peptides become progressively deglycosylated, they are rendered more sensitive to rupture by proteolytic enzymes. Thus, alternating activities of bacterial (and host) glycosidases may gradually degrade mucin macromolecules. Bacterial glycosulfatases [79] participate by removing sulfate from its attachment to galactose, GlcNAc, or GalNAc of the oligosaccharides. Since sulfate is thought to decrease the rate of mucin damage by glycosidases and proteases, those pathogens that elaborate sulfatases may accelerate mucin fragmentation [80]. A number of mucin-specific glycosulfatases have been reported in bacteria.

3.9 Mucins in Host Defense Against Intestinal Pathogens

A number of microorganisms have been found to adhere to mucin carbohydrate moieties. Protection of the intestinal epithelium against pathogenic microorganisms including, bacteria, parasites, and viruses lies in the binding capacity of mucin carbohydrates to microbial adhesins. Binding sites on mucins are thought to compete with those on underlying epithelial cells preventing attachment to the mucosal surface. In many instances, microorganisms are sloughed and swept out during peristaltic movements and defecation. Nonpathogenic organisms including the indigenous flora that reside in the adherent mucus blanket occupy an important niche within the intestine due to their ability to prevent attachment of pathogenic organisms by occupying available binding sites. The initial step in the pathogenesis of many intestinal pathogens is the binding of the

microorganism to mucin. The fate of many mucin-bound organisms lies in their ability to colonize the intestinal tract. There are four possible outcomes in the interactions between pathogens and intestinal mucins. 1: Initial mucin binding followed by elimination of the pathogen through sloughing and peristalsis, 2: Successful colonization, and the pathogen is retained in the mucus blanket and its access to the underlying epithelium is denied, 3: Colonization of the mucus layer with elaboration of virulence factors, and 4: Epithelial invasion where the mucus barrier is breached and the invading pathogen gains access to the intestinal epithelium [81]. Several pathogens including, *E. histolytica* [82], *Salmonella typhimurium* [83], *V. cholerae* [84], *Y. enterocolitica* [85], and *Candidia albicans* [86] are all known to adhere to intestinal mucin. Although the mechanisms enabling the penetration of the mucus layer by these organisms is still under investigation, release of proteinases and mucus secretagogues by pathogens may play a role in destroying the protective, polymeric nature of the mucus gel. In most cases, mucus physically traps the organisms and entangles them. Providing mucus "flushing" rates exceed bacterial colonization rates and mucin proteolytic degradation, the offending organisms are rapidly eliminated. The mechanisms used by intestinal pathogens to overcome the mucus barrier are still poorly understood and require further study.

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Section II: Manuscripts I, II, III and IV

Chapter 4: Manuscript I

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

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ABSTRACT

The adherent mucus gel layer lining the colonic epithelium is the first line of host defense against invasive pathogens, such as *Entamoeba histolytica*. The mucus layer prevents the attachment of amoeba to the colonic epithelium by trapping and aiding in the expulsion of the parasite. Disruption of the mucus 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 [^{35}S]cysteine as a substrate. Cysteine proteinases degraded mucin in a time- and dose-dependent manner. A significant reduction (>50%) in high M_r mucin with altered buoyant density was observed when degraded mucin was analyzed by Sepharose 4B column chromatography, SDS-PAGE and autoradiography, and CsCl density gradient centrifugation. Mucinase activity was abrogated by the specific cysteine protease inhibitor trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64) 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 mucus barrier by disrupting the structure of the MUC2 polymer. Mechanistically, the parasite achieves this via proteolytic degradation of the terminal cysteine-rich domains.

INTRODUCTION

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 annum [1, 2]. Although less than one percent 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: 1) colonization, 2) mucus disruption and/or depletion, and 3) binding to and cytolysis of host colonic epithelial cells. Histopathology studies in the gerbil model of invasive amebiasis suggest that amoeba first colonize the mucus layer by adherence via the parasite surface Gal-lectin to galactose (Gal) and N-acetyl-D-galactosamine (GalNAc) residues present on colonic mucin [3]. Following colonization, the parasite causes a disruption and/or dissolution of the mucus layer to gain access to the underlying epithelium. This phenomenon may be a result of the concerted actions of a battery of cysteine proteinases released by the parasite into its microenvironment [4]. The amoeba cysteine proteinases have also been implicated in the recruitment of host inflammatory cells to the site of invasion [5]. Subsequent to depletion of the mucus barrier, the parasite may come into contact with and cause lyses 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 [6].

Colonic mucin serves an important function in preventing parasite invasion of the colon. Amebic adherence to Gal/GalNAc residues of MUC2 mucin facilitates colonization of the mucus layer lining the colon via the 170 kDa Gal-lectin. This high affinity (Kd = 8.20×10^{-11} M) interaction inhibits parasite adherence to and cytolysis of target cells, in

turn, protecting the colonic epithelium from parasite invasion [7]. In order for the parasite to gain access to the underlying epithelial cells, it must first breach the protective mucus layer. The mechanisms, which enable the parasite to overcome this barrier, have yet to be determined.

MUC2 is the major glycoprotein component of the colonic mucus gel layer. The MUC2 apoprotein (**Fig. 4.1**) 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 [8, 9]. Both mucin domains are heavily glycosylated with oligosaccharides bound to serine and threonine residues via *O*–glycosidic bonds. Twenty-one separate oligosaccharide structures have been previously identified in the major colonic mucin species and characterization studies have revealed oligosaccharides ranging in chain length from two to 12 residues for the mature MUC2 glycoprotein [10]. These mucin 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.

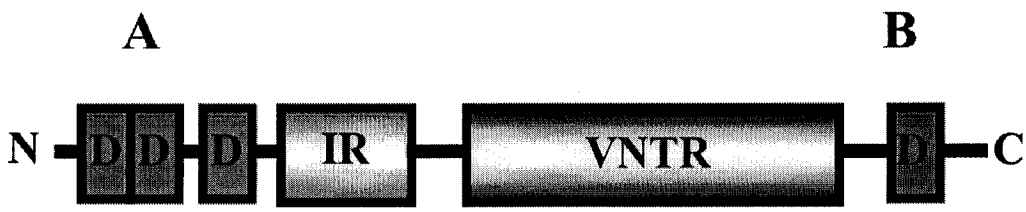

Figure 4.1. Hypothetical model of a MUC2 monomer. The molecular mass of the monomer is approximately 1.5×10^6 Da, containing ~5000 amino acids [11]. The mucin domains are represented by shaded boxes and represent the IR (180 kDa) and VNTR (~930 kDa). The protein core of the IR and VNTR are resistant to proteolytic attack due to steric hindrance. Less-glycosylated segments (A, 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.

The N- and C- terminal regions, which flank the mucin tandem repeats, are composed of various cysteine rich D-domains that share a high sequence similarity to the polymeric serum glycoprotein, von Willebrand factor [12]. The terminal D-domains are poorly glycosylated in comparison to the mucin domains. These cysteine-rich regions play a critical role in the disulphide dependent dimerization and subsequent polymerization of MUC2, which gives rise to the visco-elastic and protective nature of mucus [13, 14]. Mucus gel formation has been observed to be inhibited upon disruption of the non-glycosylated regions of mucin, either by disulphide bond reduction or proteolytic digestion [11, 15]. In addition, studies using rat MUC2, a homologue to human intestinal MUC2, have revealed these flanking regions to be extremely susceptible to proteolytic cleavage [14].

E. histolytica releases significant quantities of cysteine proteinases (EhCPs) into its environment [4]. EhCPs are the major class of proteinase produced by the trophozoite [4, 16-18] and a direct correlation between EhCP activity and amebic virulence and invasiveness has been reported [19, 20]. The EhCPs degrade extracellular matrix proteins such as laminin, collagen and fibronectin [21], contributing to the cytopathic effect involving the detachment of host epithelial cells [22]. The proteinases may play a key role in immune evasion since they have been found to degrade immunoglobulins as well as complement [23]. 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 [24]. In addition, incubation of trophozoites with the cysteine proteinase inhibitor E-64 greatly reduced liver abscess formation in severe combined immunodeficient mice [25]. 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, and consequently, this may alter its gel forming ability and abrogate its protective function. Herein, we examine the interactions between *E. histolytica* secretory proteinases and LS 174T cell mucin as a model for invasive amebiasis.

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 media (MEM) (InVitrogen Corporation, Burlington, ON) supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, UT), 100 μg of streptomycin sulfate per ml, 100 units of penicillin per ml, and HEPES. Cultures were grown in plastic tissue culture flasks (15 by 10 cm) and maintained in a humidified 5% CO_2 atmosphere at 37°C as previously described [26]. 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 cells

(CHO) were cultured in F12 media (Invitrogen) supplemented with 10% fetal bovine serum, 100 units of penicillin per liter, and 100 μg of streptomycin sulfate per ml at 37°C. Upon confluence, cells were harvested by 0.25% trypsin digestion for 5 minutes.

Cultivation and Harvesting of *E. histolytica*

HM-1:IMSS *E. histolytica* trophozoites serially passaged through gerbil livers to maintain high virulence were maintained axenically in TYI-S-33 media at 36.6°C as previously described [26]. Trophozoites were harvested at log phase growth (72 hours); the trophozoites were chilled on ice for 10 min and collected by centrifugation (700 × 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 (2×10^7 amoeba/ml) in the absence of serum for 2 hours at 36.6°C. Trophozoites were collected by centrifugation (700 × g for 5 minutes 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 albumen as a standard [27] 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 [28]. Amoeba SPs were incubated with the protease inhibitors E-64 (20 μM), Pefabloc SC (4mM), EDTA-Na_2 (0.7mM) and Pepstatin (1μM) (Roche Diagnostics GmbH, Germany) for 20 minutes at 37°C prior to the assay. The change in absorbance was monitored at 440 nm and the percentage of residual activity was determined. Specific cysteine proteinase activity was measured against benzyloxycarbonyl-L-arginyl-L-arginine-*p*-nitroanilide (Z-arg-arg-*p*NA; Bachem, Torrance, CA) as previously described with some modifications [18]. The reaction

consisted of 0.1mM substrate in reaction buffer followed by the addition of secreted proteins (50 μg). 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 minutes 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 [4].

Metabolic Labeling of LS 174T Mucin

LS 174T cells were grown to 80% confluence, and culture media was removed and replaced with fresh MEM media supplemented with [^{35}S]cysteine (0.5 μCi/ml, sp act, >1000 Ci/mMol; Amersham Biosciences, Baie D'Urfé, QC). Supernatants containing [^{35}S]cysteine labeled mucin were collected twice weekly for 2 weeks and stored at -20°C. [6-^{3}H]glucosamine labeling of mucin was achieved by replacing MEM media with fresh MEM containing [6-^{3}H]glucosamine (2 μCi/ml, sp act, 25-40 Ci/mMol; ICN, Montreal, QC). 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 otherwise specified. Supernatants were concentrated by speed vacuum or lyophilization. Particulates were removed by centrifugation (750 × g) for 10 minutes at 4°C, and supernatants were re-suspended 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 cm by 2.5 cm; Bio-Rad Laboratories, Richmond, CA) previously equilibrated with column buffer. The column was calibrated using the following molecular mass standards: blue dextran (2000 kDa) (Pharmacia, Uppsala, Sweden), thyroglobulin (669 kDa) and bovine serum albumin (68 kDa). Samples were eluted at a flow rate of 40 ml/hr and 4-ml fractions were collected. All purification steps were performed at 4°C. Aliquots (100 μL) of each fraction (1-40) were added to individual scintillation vials containing 5 ml of liquid scintillation fluid (ICN, Costa Mesa, CA.). The elution profile for radiolabeled mucin was determined by liquid scintillation counting. Fractions containing void volume (V_o)

mucin (fractions 11-18) were pooled and dialyzed for 24 hours against deionized water at 4°C. Total ^{3}H- or ^{35}S-labeled activity was determined for each fraction. To isolate native mucin, the fractions were monitored for protein (absorbance at 280 nm), and the elution profile was obtained, and 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 6-^{3}H-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, CA). A gradient was established by centrifugation of the samples at 250,000 × g for 48 hours at 4°C. The contents of the tubes were divided into eight equal fractions and each fraction was removed from the top and the density determined. Total ^{3}H- 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 protein concentration was determined.

Mucin Degradation Assays

Sepharose 4B Size Exclusion Chromatography

To determine mucinase activity, ^{35}S-labeled, S4B V_0 purified mucin (1×10^5 cpm) was incubated with EhSPs (50 μg) in 0.5 ml of DPBS (pH 7.0) for 6 hours at 37°C and fractionated by S4B chromatography (column, 30 cm by 0.75 cm) (Bio-Rad Laboratories, Richmond, CA). To determine the class of protease responsible for degrading mucin, SPs were incubated for 20 minutes prior to the assay with the following protease inhibitors: E-64 (20 μg/ml), Pefabloc SC (0.5 μg/ml), and Pepstatin (0.7 μg/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.

SDS-PAGE and Autoradiograph 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. Secretory products were also incubated with E-64 (100 μM) for 20 minutes prior to the assay to inhibit cysteine proteinase activity. The samples were concentrated and re-suspended in SDS-PAGE loading buffer (50 mM Tris-Cl (pH 6.8), 10 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol). Digests were analyzed by SDS-PAGE (4% stacking, 7% resolving) under reducing conditions and visualized by autoradiography by exposing the Kodak XAR-5 film with an intensifying screen to the gel for one week at -70°C) as previously described [29]. 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).

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 hours at 37°C. Specificity for CPs was demonstrated by pre-incubating the SPs with E-64 (100 μM) for 20 minutes prior to the assay. The digests were concentrated and re-suspended 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, ^{3}H-labeled V_0 mucin (10^6 cpm) was incubated with SPs (250 μg) at 37°C for 18 hours. ^{3}H-labeled mucin degradation was analyzed as described 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 [7]. 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 followed by incubation with media alone, media and S4B V_0 mucin (100 μg/ml), or mucin preincubated with SPs for 1 at 37°C. To determine if cysteine proteinases 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 (10^4) trophozoites was added to 2 × 10^5 CHO cells in M199s (volume, 1 ml). The samples were pelleted by centrifugation at 600 × g for 5 minutes at 4°C, followed by incubation at 4°C for 2 hours. 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 (mean ± 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, Pepstatin, and EDTA were used to determine the major catalytic classes of enzymes released by the parasite. In addition, other protease inhibitors (leupeptin, Phenylmethylsulfonyl fluoride [PMSF], apotinin, and *N* α-*p*-tosyl-L-lysine chloromethyl ketone [TLCK]) were used to confirm the results. The majority of the secreted protease activity against azocasein was inhibited by E-64 (> 90%) (**Fig. 4.2**). 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 (**Fig. 4.3**).

To determine the activity and specificity of the cysteine proteinases, Z-Arg-Arg-*p*NA was used as a substrate (**Table 4.1**). 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 inhibited enzyme activity, confirming the presence of cysteine proteinase activity in the SPs.

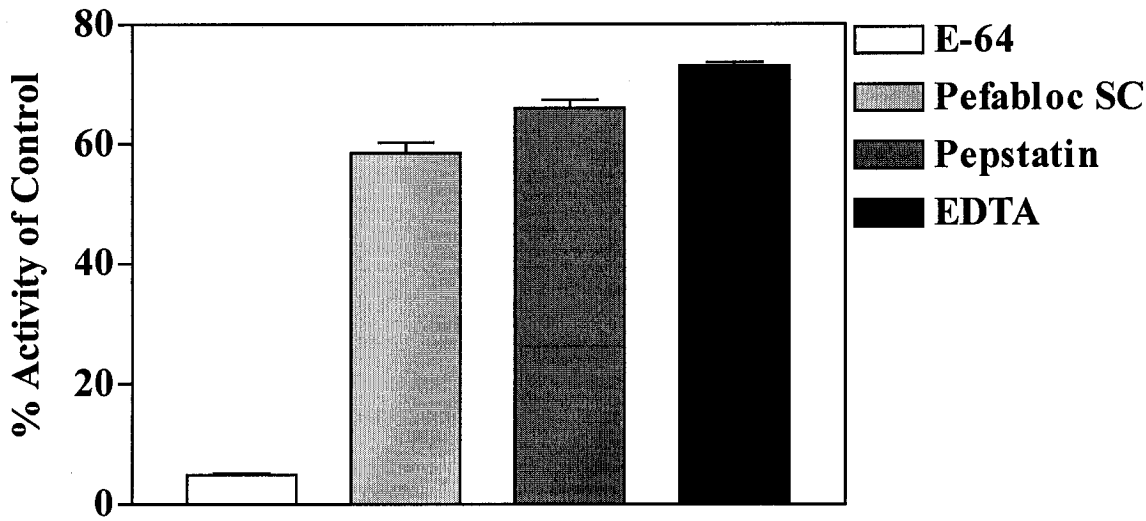

Figure 4.2. The major classes of proteolytic enzymes released by *E. histolytica.* Amoeba secretory/excratory products (50μg) were assayed for total proteolytic activity using azocasein (4%) as substrate. Secreted proteases were incubated with the following protease inhibitors: E-64 (20 μM), Pefabloc SC (4 mM), Pepstatin (1 μM), and EDTA-Na_2 (EDTA, 0.7 mM) for 10 minutes prior to the assay. The reaction was terminated by addition of 10% TCA (500μl) and precipitated protein was removed by centrifugation (3,000 × g for 5 minutes). Protease inhibition was plotted as the percent of residual activity after 2 hours of incubation compared to the control (no inhibitor). The data indicate the mean ± standard deviation (n = 3) of a representative experiment that was repeated twice.

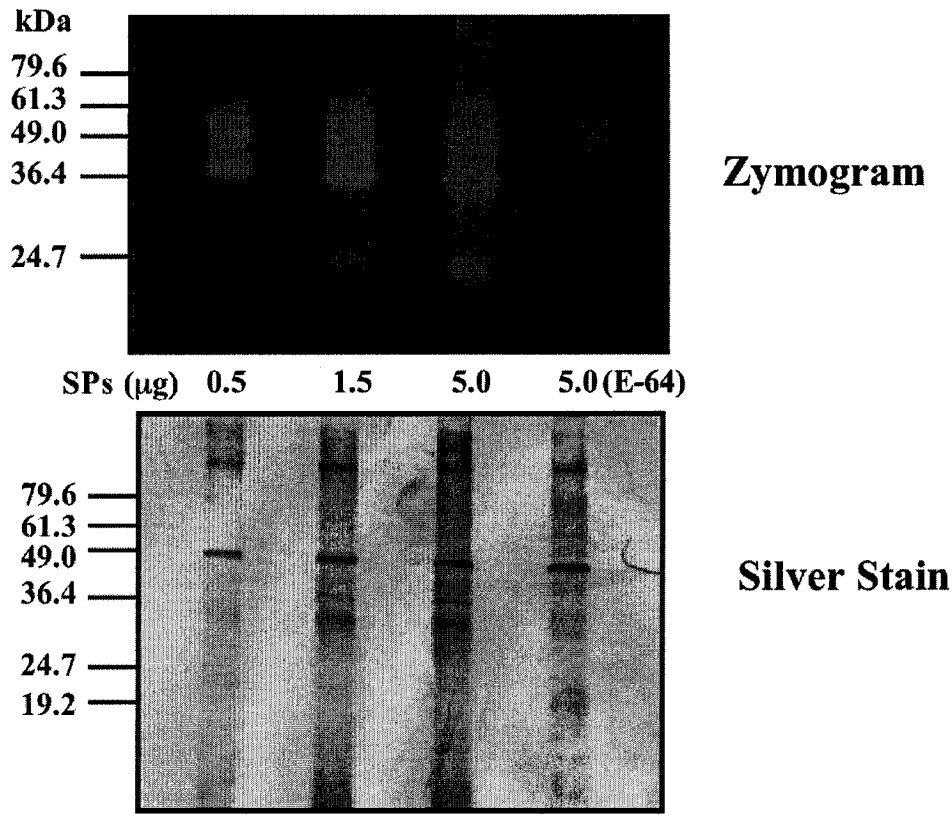

Figure 4.3. Substrate gel electrophoresis of *E. histolytica* secretory/excretory products. *E. histolytica* secretory products were separated on a 12% SDS polyacrylamide gel co-polymerized with 0.1% gelatin. Numbers indicate the amount of protein separated in μg and E-64 indicates pretreatment of proteins with 100 μM of inhibitor for 20 minutes prior to separation. Following separation of the proteins, the gel was washed with 2.5% Triton X-100 for 1 h to remove the SDS. The gel was then incubated at 37°C for two hours in reaction buffer (100mM Potassium phosphate, 20 mM DTT, [pH 7.0]). Secreted products were also separated using a 12 % SDS polyacrylamide gel and visualized by silver staining using the Bio-Rad silver stain kit according to the maufacturer's instructions.

Table 4.1. Effects of protease inhibitors on *E. histolytica* cysteine proteinase activity*.

Inhibitor	Inhibitor Class	% Residual Activity
Cystatin	Cysteine	2 ± 0.79
E-64	Cysteine	5 ± 0.54
Leupeptin	Cysteine/Serine	5 ± 0.43
TLCK	Cysteine/Serine	2 ± 0.25
TPCK	Cysteine/Serine	6 ± 1.49
Pefabloc-SC	Serine	93 ± 1.43
Aprotinin	Serine	100 ± 2.51
Pepstatin	Aspartic	100 ± 7.57
Phosphoramidon	Metallo-	100 ± 1.00

*Secreted cysteine proteinase activity was measured using the synthetic substrate Z-Arg-Arg-pNA. *E. histolytica* cysteine proteinases are known to cleave substrates with arginine in the P_2 position. Class specific protease activity was determined by incubation of the secreted products with protease inhibitors for 10 min prior to the assay. The assay was performed as described in methods. Results are given as the mean percent residual activity (of control) ± SD ($n = 3$) and the experiment was repeated three times.

Degradation of Mucin by *E. histolytica* Cysteine Proteinases

Analysis by Sepharose 4B Gel Filtration

High-molecular-weight (MW) mucin polymers can be partially isolated using S4B column chromatography [26]. As shown in **Fig. 4.4 A**, [^{35}S]cysteine labeled mucin eluted exclusively in the V_0, whereas mucin incubated with 50μg 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 12-25. To determine if cysteine proteinase activity was responsible for mucin degradation, SPs were pretreated with protease inhibitors. Consistent with the data in **Table 4.1**, 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. 4.4 B**, 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.

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. **4.5 A**, SPs degraded mucin in a time-dependent fashion. Mucinase activity occurred as early as 15 min and increased 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 (arrows). 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. 4.5 B**). 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 the cysteine proteinase inhibitor E-64, clearly implying CPs disrupt the polymeric structure of MUC2.

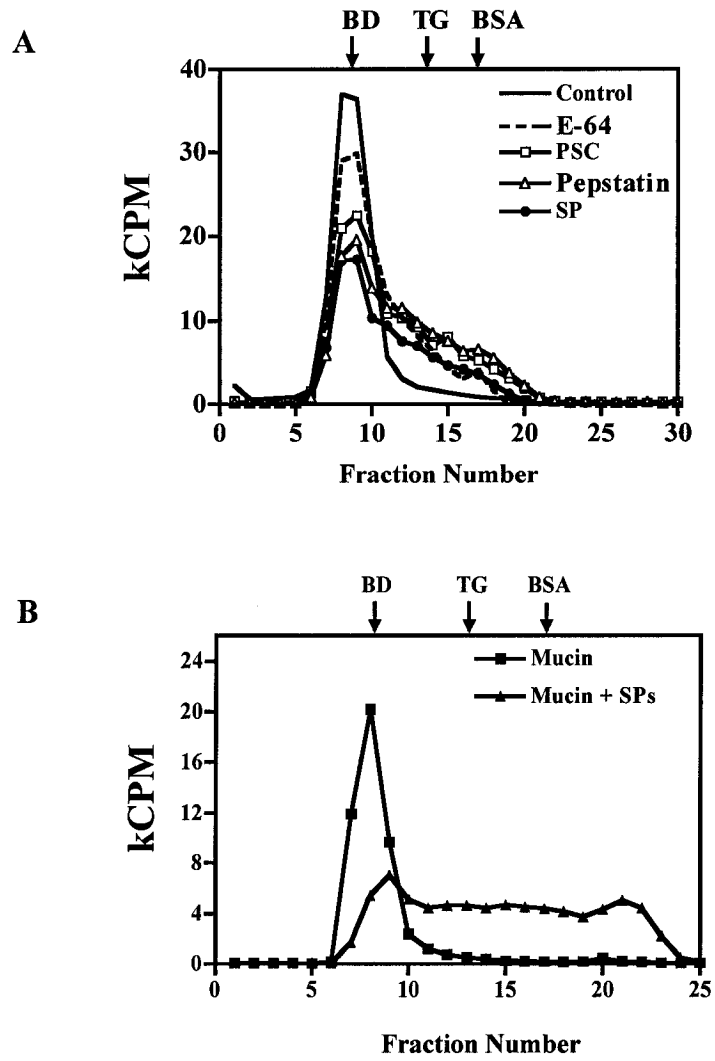

Figure 4.4. (A) S4B elution profile of [^{35}S]cysteine-labeled mucin degraded by *E. histolytica* SPs. The elution profiles of control, high-MW mucin and mucin incubated with SPs alone or with E-64, Pefabloc SC (PSC), or pepstatin are shown. kCPM, 1,000 cpm. (B) S4B elution profiles of CsCl mucin (fraction 6) degraded by *E. histolytica* SPs. The elution profiles of control, [^{35}S]cysteine-labeled mucin alone and mucin incubated with SPs are shown. For details of the molecular mass markers, see methods (BD, blue dextran; TG, thyroglobulin).

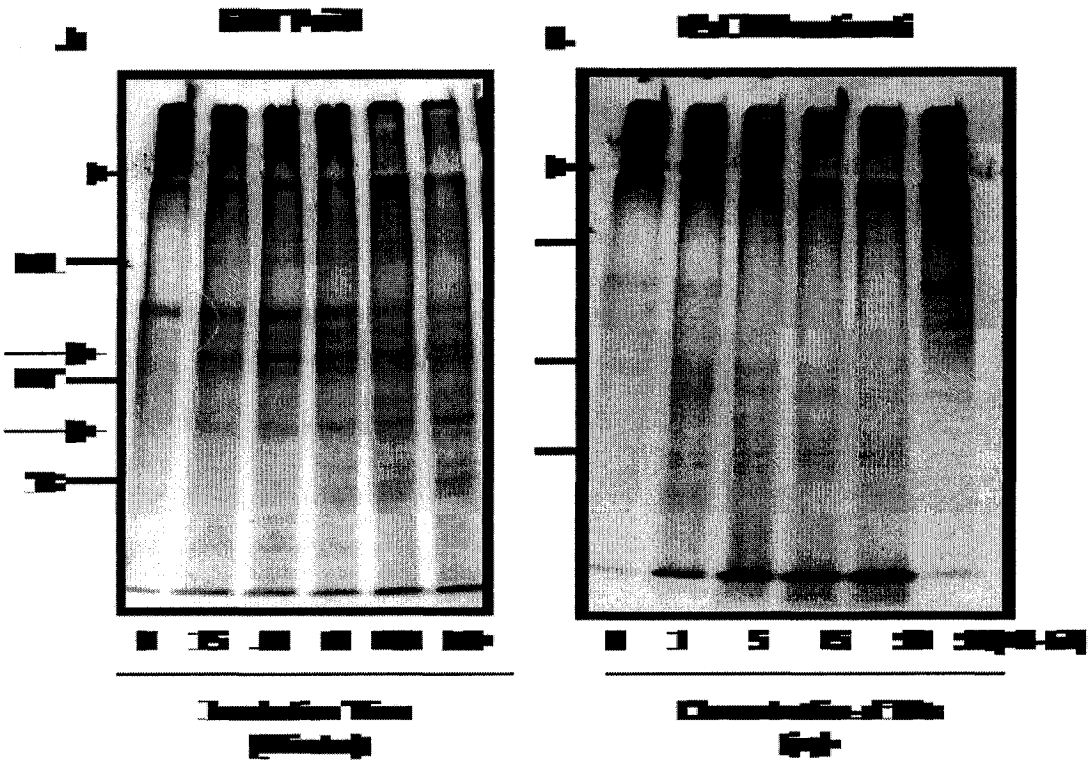

Figure 4.5 (A) Time-dependent degradation of [^{35}S]cysteine-labeled S4B V_0 mucin incubated with SPs (50 μg). The positions of molecular mass markers (in kilodaltons) are indicated to the left of the gel. (B) Dose-dependent degradation of CsCl-purified, ^{35}S-labeled mucin (fraction 6). In the rightmost lane, SPs were preincubated with E-64. The positions of the 4% separating gel (arrowheads) and cleavage products (arrows) are indicated.

Analysis by Cesium Chloride Density Centrifugation

Purification of mucin by CsCl density gradient centrifugation separates the noncovalently bound mucins from other proteins in the high-density hexose-rich fractions [26]. **Fig. 4.6 A** clearly shows that the majority of the [^{35}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 fractions 6 on a CsCl density gradient [26]. In contrast, following incubation with SPs, there was a dramatic shift in ^{35}S-labeled mucin from fraction 6 to fractions 1 to 4, of lower buoyant density (<1.40 g/ml; **Fig. 4.6 B**). The appearance of ^{35}S-activity 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. 4.6 C**, where pre-treatment of SPs with E-64 prior to the assay, inhibited the degradation of MUC2, and resulted in a notable reduction in degraded mucin in fractions 1-3 and an increase in ^{35}S-activity in fractions 5-7.

[6-^{3}H]glucosamine-labeled mucin displayed a similar partitioning profile compared to ^{35}S-labeled mucin; however, the majority of the 6-^{3}H-labeled mucin remained in fraction 6 after exposure to SPs, suggesting proteinase but not glycosidase activity (data not shown).

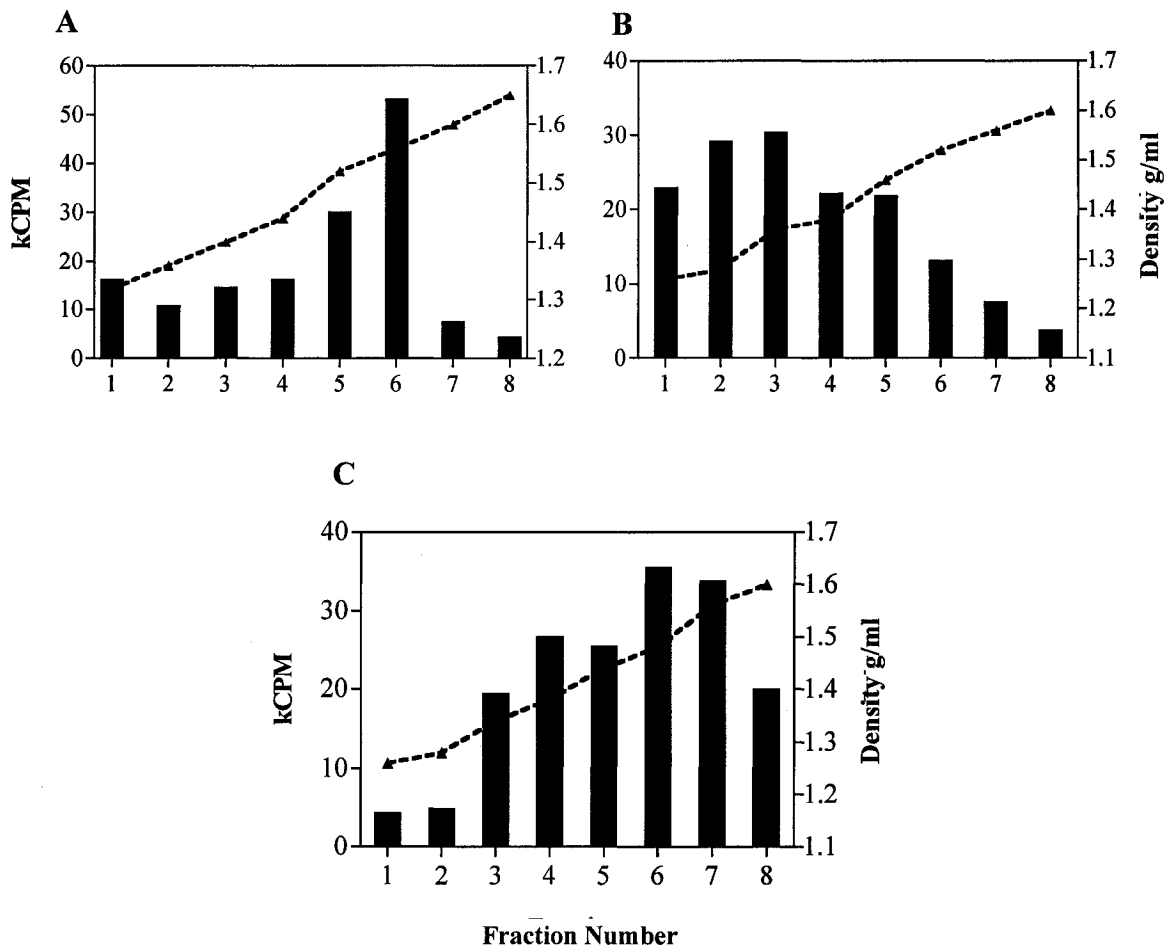

Figure 4.6 (A) CsCl density gradient centrifugation of mucin degraded by *E. histolytica* SPs. Mucin partitioned in fraction 6, with a density > 1.42 g/ml. (▲--▲). (B and C) Mucin incubated with SPs (B) and SPs pretreated with E-64 (C). Results are displayed as a representative graph of three separate experiments. kCPM, 1,000 cpm.

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. 4. 7**, 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 μg and 250 μg of SPs, amebic adherence to target cells increased 52 and 71%, respectively. To examine the role of cysteine proteinases in this event, SPs (250 μg) were pre-incubated with E-64. Interestingly, not only was E-64 found to inhibit mucin degradation, but it also helped to maintain the protective function of the mucin. This was evident due to 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.

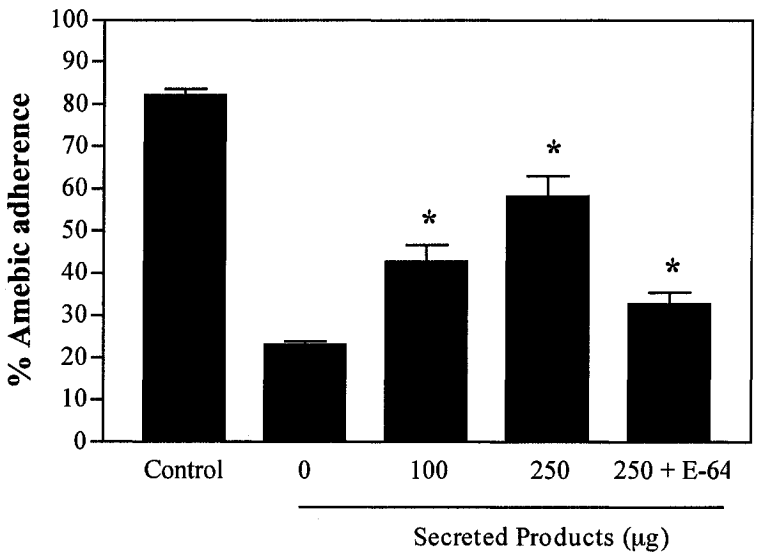

Figure 4.7. *E. histolytica* cysteine proteinases alter the protective function of LS 174T cell mucin. Note that preincubation of SPs with E-64 (100 μM), significantly reversed amebic adherence to target cells (values that were significantly different [*p<0.05] from the value for the homologous control are indicated by the asterisks). The mean amebic adherence of different concentrations of SPs ± SD (error bar) (n = 6) from one representative experiment of three experiments is shown.

DISCUSSION

The majority of individuals infected with *E. histolytica* are asymptomatic carriers, however, invasion does occur in a small percent of those afflicted with the parasite [30]. In order for invasion to occur, the parasite must overcome the protective mucus layer lining the colon. Histopathology studies of the human colon and rectum have revealed that the mucus layer lining these regions is separated into two striated layers. The outer layer contains the majority of bacteria and fecal content and the inner layer contains little to no bacteria [31]. 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 mucus blanket by inducing goblet cell hypersecretion prior to invasion [32]. In this study, we have show 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 [26]. Mucin collected from CsCl density gradients has been extensively characterized and shown to be free of contaminants such as proteoglycans or low-MW proteins [26]. Metabolic labeling of LS 174T cell mucin with [^{35}S]cysteine allowed the tracking of 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 [33]. This indicates the importance of disulphide bond mediated mucin polymerization in mucus gel formation.

Degraded mucin was not as effective at inhibiting amebic adherence to target cells as the native 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 mucus 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 in our study, their role as virulence factors cannot be entirely ruled out. One could speculate that differences in the length 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 mucus 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 postinvasion of the protective mucus 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* [34]. A study has shown that out of these organisms, only the trichomonads produced the necessary range of glycosidases needed for the complete breakdown of mucin [35]. This may suggest that the other organisms utilize an alternative method for overcoming the mucus barrier. At least seven genes encoding for cysteine proteinases have currently been identified in *E. histolytica* [36-38]. However, only gene products from five of these genes, *EhCP1, EhCP2, EhCP3, EhCP5*, and *EhCP112* have been identified in cultured trophozoites [36, 37-39]. Bruchhaus *et al.* [37], have reported that the enzymes EhCP1, EhCP2 and EhCP5 contribute to approximately 90% of the total cysteine proteinase

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.

Acknowledgments

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Connecting Statement I

In Manuscript I, we set out to characterize the interactions between *E. histolytica* and colonic mucin. We have identified the *E. histolytica* cysteine proteinases as the major class of enzyme responsible for degrading colonic mucin. In addition, we also discovered that the parasite proteinases decrease the cytoprotective effect of mucin. In the next series of experiments in Manuscript II, we characterized the involvement of the cysteine proteinases in mucus gel penetration and epithelial cell invasion using our LS 174T cell model of colonic invasion. The generation of cysteine proteinase deficient parasites was achieved by antisense technology and allowed us to directly examine the role of the proteinases in mucus gel disruption.

Chapter 5: Manuscript II

Antisense Inhibition of *Entamoeba histolytica* Cysteine Proteinases Inhibits Colonic Mucus Degradation*

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ABSTRACT

Background & Aims: Cysteine proteases are believed to be major virulence factors released by *E. histolytica* in the pathogenesis of intestinal amebiasis. However, the exact role these proteases play in overcoming the protective mucus barrier, as a prerequisite to epithelial cell disruption is not known. Herein, we determined whether *E. histolytica* trophozoites expressing the antisense transcript to *cysteine protease 5* (*EhCP5*) could degrade colonic mucin and destroy epithelial cells. **Methods:** Cysteine protease deficient amoebae were generated by antisense inhibition of *EhCP5*, and assayed for proteolytic activity against LS 174T [^{35}S]cysteine labeled mucin and analyzed by SDS-PAGE and Sepharose 4B chromatography. Mucinase activity of recombinant EhCP5 was determined using purified colonic mucin. Disruption of an intact mucus barrier and epithelial cell invasion by amoebae were measured using LS 174T monolayers with an intact mucus gel and CHO cells devoid of a mucus barrier. **Results:** Trophozoites with reduced cysteine proteinase activity were ineffective at degrading [^{35}S]cysteine labeled colonic mucin compared to wild-type amoebae by >60%. However, bioactive recombinant EhCP5 degraded >45% of purified native mucin which was specifically inhibited by the cysteine proteinase inhibitor, E-64. Cysteine protease deficient trophozoites could not overcome a protective intact mucus barrier and disrupt an LS 174T cell monolayer; however, they readily adhere to and disrupt CHO monolayers devoid of a mucus barrier. **Conclusions**: These findings unravel a central role for *E. histolytica* cysteine proteinases as key virulence factors in disrupting an intact mucus barrier in the pathogenesis of intestinal amebiasis.

INTRODUCTION

Gastrointestinal mucus acts as the first line of host defense against enteric parasites by preventing the attachment of microorganisms to enterocytes and directly influences the ability of pathogens to colonize the gastrointestinal tract. In addition, mucin also aids in the expulsion of intestinal microbes [1-3]. MUC2 is the major gel-forming mucin secreted in the colon and the monomer has a mass of ~1.5 million Daltons [4]. The oligosaccharide component accounts for up to 90 % of the dry weight of the molecule and this dense glycosylation protects the mucin domains from proteolytic cleavage and is responsible for microbial binding and colonization.

MUC2 forms polymers, which upon hydration give rise to the viscoelestic and protective properties of the mucus gel. The ability of MUC2 to form a gel is dependent upon its nature to polymerize via the cysteine-rich D-domains by forming intramolecular disulfide bonds with corresponding termini of MUC2 monomers [4-6]. These domains are poorly glycosylated in comparison to the mucin domains and therefore are hypothesized to be vulnerable to proteolytic attack by enzymes released by invasive enteric pathogens. Breaching of the mucus barrier is a prerequisite to epithelial cell attachment by invasive microorganisms, and a limited number of studies have attempted to dissect the mechanisms used by pathogens to overcome the mucus layer during the course of infection. Degradation of the protein and/or oligosaccharide components of mucin by enteric pathogens is thought to be at least one strategy used by these organisms to weaken and traverse the mucus gel. Motility, as well as expression of the Zn^{2+}-dependent metalloprotease Hap (mucinase) by *Vibrio cholerae* is necessary for the bacteria to translocate through intestinal mucus [7]. The enteric pathogens *Candida albicans*, *Yersinia enterocolitica, Shigella flexneri,* and *Helicobacter pylori* all produce virulence factors involved in mucus degradation [8-11].

Entamoeba histolytica is the etiological agent of human amebiasis, and the motile trophozoite form of the parasite colonizes the large bowel and invades the colonic epithelium resulting in mucosal damage and colitis. In the most severe cases of amebiasis

the trophozoites enter the blood stream while invading mucosal tissue to cause liver abscesses, resulting in death if left untreated. Cysteine proteinases produced by the parasite are directly involved in tissue invasion through their ability to degrade extracellular matrix proteins, and play a key role in immune evasion by degrading host antibodies and complement [12-14]. We have previously shown that *E. histolytica* secreted products effectively degrade colonic mucin and the degraded mucin could not inhibit amebic adherence to target cells as well as the native molecule [15]. These results suggest that the polymeric structure of mucin must be maintained to protect the epithelium. The molecules involved in mucus degradation have not yet been identified, but cysteine proteases are likely to be the key virulence factors involved in disrupting the mucus barrier. Since breaching the mucus barrier is a prerequisite for invasion by the parasite, proteolytic cleavage of mucin by cysteine proteinases would be sufficient to disrupt the polymeric nature of mucin and permit the parasite to make contact with the colonic epithelium. In this study, we specifically determined whether *E. histolytica* cysteine proteinases could disrupt colonic mucin by transfecting the parasite with the pSA8 plasmid expressing antisense to *EhCP5* to generate cysteine proteinase deficient amoebae. This enabled us to directly examine the involvement of the cysteine proteinases in the initial events of invasive amebiasis using colonic epithelial cells that produce a protective mucus barrier. Our results show that the cysteine proteinases are essential virulent components that abolish the cytoprotective function of mucin, enabling the parasite to bind and destroy colonic epithelial cells.

METHODS

Cell Culture and Preparation of *E. histolytica* Secretory Components

LS 174T cells (ATCC, Rockville, MD, USA) were cultured to 70-80% confluence in minimal essential medium (MEM) (Invitrogen, Burlington, Ontario, Canada) supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, Utah, USA), 100 μg of streptomycin sulfate per ml, 100 U of penicillin per ml, and 20 mM HEPES [15, 16]. CHO cell cultures were maintained in F12 medium (Invitrogen) supplemented with 10% fetal calf serum, 100 μg of streptomycin sulfate per ml, 100 U of penicillin per ml,

and 20 mM HEPES (Invitrogen). *E. histolytica* HM-1:IMSS trophozoites were serially passaged through gerbil livers to maintain high virulence and were cultured axenically in TYI-S-33 medium at 36.6°C as previously described [16]. Trophozoites were harvested after 72 hours at logarithmic growth phase by incubation on ice for 10 minutes followed by centrifugation (700 × *g*) for 5 minutes at 4°C. Secreted products were collected from trophozoites (2×10^7/ml) incubated in Hank's balanced salt solution (Invitrogen) at 37°C for two hours as described elsewhere [17].

Transfection of *E. histolytica* Trophozoites

Trophozoites in logarithmic growth phase were transfected with the pSA8 (*EhCP5* antisense) or pEhAct-neo (parental) plasmids as previously described using a Bio-Rad gene Pulser [18]. Briefly, following collection, the trophozoites were washed twice with phosphate-buffered saline followed by one wash with cytomix (120 mM KCl, 0.15mM $CaCl_2$, 10mM potassium phosphate buffer, [pH 7.5], 25 mM HEPES, 2 mM EGTA, and 5mM $MgCl_2$). The parasite was resuspended in cytomix to a concentration of 3×10^6 amoeba/ml with 2.5 μg of DEAE dextran and 100 μg of plasmid (pSA8 or pEhAct-neo) for transfection in 0.4 cm electroporation cuvettes (Bio-Rad). The transfected parasites were allowed to recover for 48 hours prior to drug selection with G-418 sulfate (G418) (Invitrogen). The concentration of G418 was raised to 60 μg/ml over a period of four weeks.

Southern and Northern Blot Analysis

Total nuclear DNA was isolated from *E. histolytica* trophozoites as previously described [19]. The digested DNA was then subjected to electrophoresis through a 1% agarose gel, transferred to a Hybond-N nylon membrane (Amersham Biosciences, Baie d'Urfé, Québec, Canada) and fixed by UV radiation. The blots were probed under stringent conditions with a [α-^{32}P]dCTP (ICN Biomedicals Inc., Irvine, CA, USA) labeled DNA fragment (877bp) of the *E. histolytica EhCP5* gene amplified from genomic DNA [20]. For Northern blot analysis, RNA from *E. histolytica* was isolated with TRIZOL reagent (Invitrogen). Probes were generated by PCR amplification of *E. histolytica actin* and *EhCP5* genes, as well as the neomycin phosphotransferase gene (*neo*) of bacterial origin

[20]. *EhCP5* sense and antisense [α-^{32}P]dUTP (ICN Biomedicals Inc.) labeled probes were generated by *in vitro* transcription of *EhCP5* in the pGEMT-easy vector (Promega Corporation, Madison, WI, USA) using SP6 and T7 polymerases. The blots were hybridized with the probes and the optical density of the bands was analyzed using NIH image software (http://rsb.info.nih.gov/nih-image/).

Cysteine Protease Activity and Expression of Recombinant EhCP5

Cysteine proteinase (CP) activity was measured in *E. histolytica* total cell lysates prepared by three freeze-thawing cycles. One unit of protease activity was defined as the μmol of substrate digested per min mg^{-1} protein [21]. Protease activity was also monitored by zymogram analysis with a 12% polyacrylamide gel copolymerized with 0.1% gelatin (Sigma-Aldrich, Oakville, Ontario, Canada) as described elsewhere [22]. EhCP5 was expressed in *E. coli* strain BL21(DE3) [pAPlacIQ] using the expression vector pJC45. The recombinant protein was expressed as an insoluble histidine-tagged proenzyme and was solulibilized, purified, and refolded as described elsewhere [23]. Processing of the recombinant enzyme to the mature active form was monitored by gelatin zymogram gel analysis and cleavage of the synthetic substrate z-Arg-Arg-pNA [21].

Measurement of Amebic Adherence to Epithelial Cells

Adherence of *E. histolytica* to CHO cells was performed using a standard protocol as previously described with modifications [24]. Trophozoites were washed in M199s media (Invitrogen) supplemented with 5.7 mM cysteine, 25 mM HEPES and 0.5% BSA (Sigma-Aldrich). The CHO cells were resuspended in media and incubated at a concentration of 20:1 with amoebae in a total volume of one ml of M199s. The cells were pelleted by centrifugation at 600 × g for 5 minutes at 4°C and incubated at the same temperature for 2 hours (hrs). Rosette formation is defined as the percentage of amoebae adherent to three or more CHO cells and was determined by counting > 100 amoebae per condition from a total of six tubes per condition performed 3 times.

Purification of [^{35}S]cysteine Labeled Mucin from Colonic Cells and Mucin Degradation Assays

Labeling of mucin with [^{35}S]cysteine allows for tracking of the poorly glycosylated regions of MUC2. For the preparation of radiolabeled mucin, LS 174T cells were grown in medium containing 2 μCi/ml of [^{35}S]cysteine (specific activity > 1000 Ci/mmol; MP Biomedicals, Irvine, CA, USA) and the secreted mucin was purified as described previously [15, 16]. Labeled mucin was incubated with parasite cell lysate, secreted products or recombinant EhCP5 in DPBS at 37°C. For analysis by SDS-PAGE, the samples were concentrated and resuspended in loading buffer (50 mM Tris-HCl [pH 6.8], 10 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol) and separated by SDS-PAGE with a 4% stacking and a 7% running gel. The dried gels were exposed to Kodak ZAR-5 film with an intensifying screen for one week at –70°C. The degradation was measured by densitometric analysis of high molecular weight stacking gel mucin as previously described using the NIH Image software (http://rsb.info.nih.gov/nih-image/) [15, 25]. Additional analyses of the digests were also conducted by Sepharose 4B gel filtration (column; 30 cm × 0.75 cm, Bio-Rad Laboratories Ltd.) and 30 fractions of 0.5 ml were collected and subjected to liquid scintillation counting [15].

Epithelial Monolayer Invasion Assay

LS 174T and CHO cell monolayer destruction assays were performed with wild type amoebae as well as pEhActNeo and pSA8 transfectants. Cell lines were seeded onto 24 well plates (Corning, NY, USA) and grown to 80% confluency. *E. histolytica* trophozoites were resuspended in M199s media supplemented as above, to a concentration of 1×10^5 amoebae/ml and when stated, parasites were incubated with 100 μM E-64. The monolayers were washed three times with DPBS (37°C) and one ml of the trophozoite suspension was added to each well. The cells were incubated at 37°C for 3 hrs to assess CHO cell monolayer destruction and 1, 3, 4, and 6 hrs for LS 174T monolayer destruction assays. Trophozoites were removed by incubation at 4°C and by washing the monolayers three times with ice cold DPBS. The remaining inact monolayers were fixed with 2.5% glutaraldehyde (Sigma-Aldrich) in DPBS and stained with 0.1% methylene blue in 100 mM borate buffer [pH 8.0]. The stain was extracted from the cells

in one ml of 1N HCl and incubation at 37°C for 30 minutes. The absorbance for each well was measured at 660 nm and the amount of destruction was determined by calculating [A_{660} control wells – A_{660} experimental wells] / [A_{660} control wells] × 100 [16]. Survival of amoebae was determined using the trypan blue exclusion assay.

LS 174T Cell Mucin Secretion

LS 174T cells were incubated with 1 μCi/ml [6-^{3}H]glucosamine hydrochloride (40 Ci/mmol; MP Biomedicals Inc. Irvine, CA, USA) for 48 hrs and washed three times with MEM medium prior to addition of test substances. Epithelial cells grown on 24 well plates were incubated with 20 μM calcium ionophore A 23187 (Sigma-Aldrich), secreted products from *E. histolytica,* M199s medium, or whole trophozoites (1×10^5) separated by a Millicell-HA culture plate insert with a pore size of 0.45 μm (Millipore, Bedford, MA, USA). Secreted ^{3}H-activity was determined for 100 μl aliquots of medium collected after four hrs of incubation by liquid scintillation counting as previously described [15]. The secreted mucin was analyzed by Sepharose 4B gel filtration as described above.

Statistical Analysis

Data (mean ± SD) were analyzed using the student *t* test with Prism 4 (GraphPad Software Inc. San Diego, CA.) A *P* value of $< 0.05\%$ was considered statistically significant.

RESULTS

Antisense Inhibition of EhCP5 Decreases *E. histolytica* Cysteine Protease Activity

To determine whether *E. histolytica* trophozoites were successfully transfected with the pSA8 plasmid or the parental pEhAct-neo plasmid, Southern blot analysis was performed on trophozoite total genomic DNA. Genomic DNA isolated from the pEhAct-neo transfectants hybridized with the *EhCP5* probe to reveal a band corresponding with the genomic copy of the gene, while pSA8 genomic DNA hybridized with the probe to reveal two bands, one corresponding to the genomic copy and the other, the episomal *EhCP5* gene of plasmid origin (**Fig. 5.1 A**). Northern blot analysis confirmed that *EhCP5* antisense transcripts were expressed in the pSA8 transfectants and were not present in the control transfectants (**Fig. 5.1 B**).

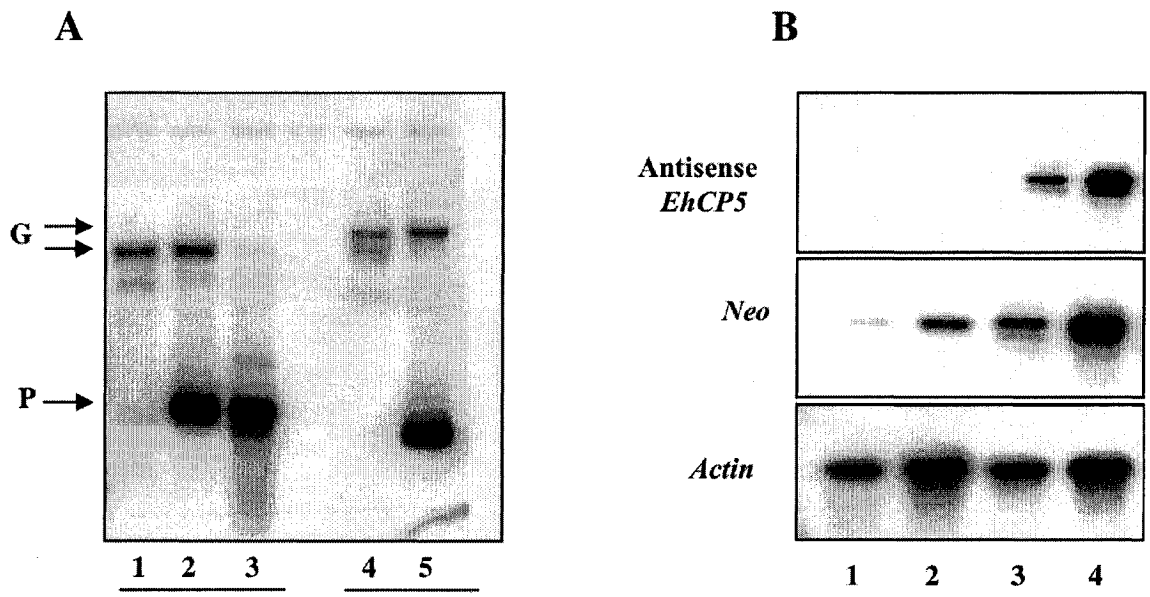
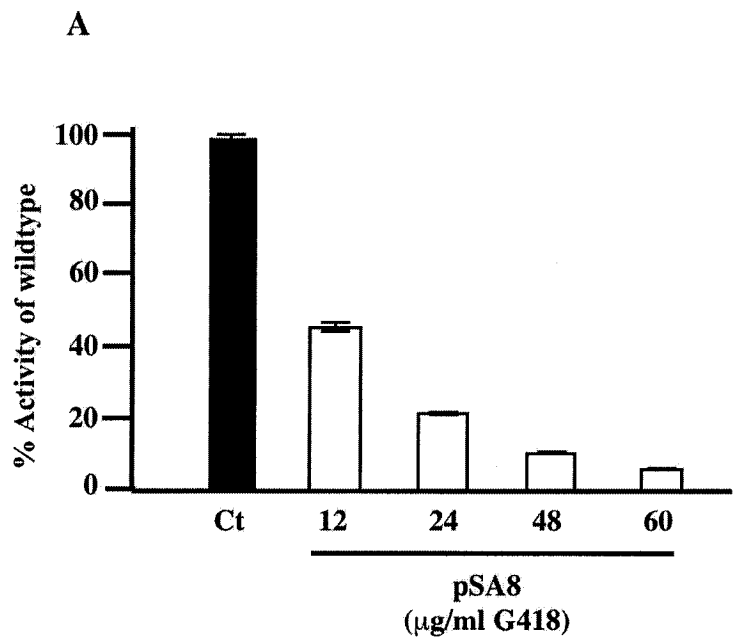


Figure 5.1. Characterization of *E. histolytica* transfectants

(A) Southern blot analysis of *E. histolytica* transfectants. 10 μg of total DNA from pSA8 and pEhAct-neo transfectants grown in G418 was digested with the restriction enzyme pairs *EcoR* I and *Sal* I (lanes 1-3) or *Bgl* II and *Sal* I (lanes 4-5) (Invitrogen). (1) pEhAct-neo transfectants grown in 6 μg/ml G418, (2) pSA8 grown in 6 μg/ml, (3) pSA8 grown in 60 μg/ml (4) pEhAct-neo in 6 μg/ml and (5) pSA8 in 6 μg/ml. (G) Genomic copy of

EhCP5. (P) Plasmid derived *EhCP5* gene. (B) Northern blot analysis. 10 μg of total RNA from amoebae was denatured using glyoxyl and was subjected to electrophoresis through a 1% agarose gel and hybridized with the genes encoding actin, neomycin phosphotransferase, and EhCP5. *E. histolytica* pEhAct-neo transfectants grown in (1) 6 μg/ml and (2) 60 μg/ml of G418. pSA8 transfectants grown in (3) 6 μg/ml and (4) 60 μg/ml of G418.

Moreover, antisense expression increased in trophozoites grown in high concentrations of G418, and total cysteine proteinase activity against z-Arg-Arg-pNA was reduced by ~90% in both the pSA8 transfectants grown in 48 or 60 μg/ml of G418 (**Fig. 5.2 A**). Gelatin zymogram analysis revealed an evident decrease in proteolytic activity in the pSA8 transfectants (**Fig. 5.2 B**). *EhCP5* antisense mRNA inhibited *EhCP5* as well as the expression of other cysteine proteases which may be due to a high degree of sequence homology and conservation of all residues critical for protease function [26, 27].



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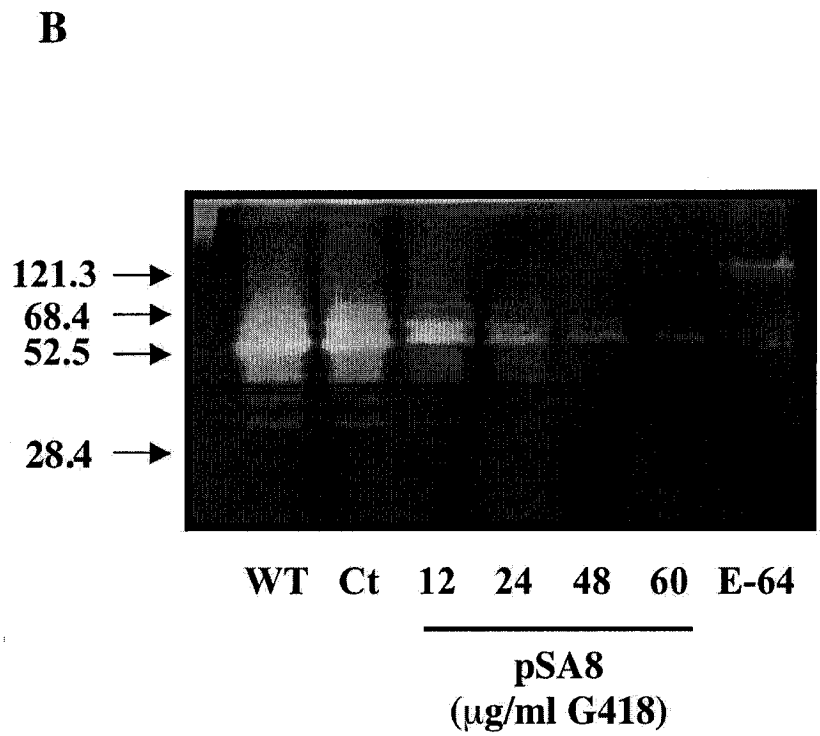


Figure 5.2 Enzymatic activity of pEhAct-neo and pSA8 transfectants

(A) Protease activity of the pEhAct-neo (Ct) and pSA8 strains was plotted as percent activity of the wild-type trophozoites. The activity was measured by monitoring the digestion of the chromogenic substrate z-Arg-Arg-pNA. (B) Gelatinase activity of wild type (WT), pEhAct-neo (Ct), and pSA8 strains grown in 12, 24, 48 and 60 μg/ml of G418. Incubation of WT lysate with E-64 (E-64).

E. histolytica Cysteine Proteases Degrade Colonic Mucin

We have previously shown that mucin degradation by *E. histolytica* secreted components was markedly inhibited by the cysteine protease inhibitor E-64 [15]. To specifically determine the role of cysteine proteinases in mucus gel disruption and epithelial cell invasion, we examined whether pSA8 transfected amoebae could degrade colonic mucin. Due to the high molecular weight and abundant glycosylation of mucin, the native molecule remains in the 4% stacking gel when separated by SDS-PAGE, allowing degradation to be calculated by densitometric analysis of radiolabeled mucin. **Figure 5.3 A**, shows that the pEhAct-neo transfectants readily degrade the [^{35}S]cysteine labeled mucin N-and/or C-terminal flanking regions similar to the wild type amoebae (100% degradation). In contrast, the pSA8 strain showed a marked decrease in mucinase activity with trophozoites grown in 24, 48, and 60 μg/ml of G418 degrading high molecular weight mucin by only approximately 20% compared to the control transfectants (**Fig. 5.3 A**). Interestingly, pSA8 transfectants grown in 24 μg/ml of G418 with < 25% of wild type cysteine proteinase activity showed almost a complete inhibition in mucinase activity similar to that of the pSA8s grown in higher concentrations of G418. This correlates with a major reduction in cysteine protease activity observed when transfectants are grown in concentrations of G418 greater than 12 μg/ml. Degradation products were also analyzed by Sepharose 4B gel filtration (**Fig. 5.3 B**). Native mucin collected from LS 174T cells elutes in the void volume (V_o) of a Sepharose 4B column calibrated with blue dextran (fractions 6-11). Incubation of labeled mucin with pEhAct-neo or wild type lysate resulted in a net reduction of ^{35}S-labeled V_o mucin and a subsequent increase in lower molecular weight mucin fragments in the included fractions (fractions 12-20). pSA8 transfectants exhibited a significant loss of mucinase activity as seen by high levels of intact mucin eluting in the V_o and a lack of degradation fragments, indicative of cysteine proteinase mediated disruption of the N- and/or C- terminal flanking regions of mucin.

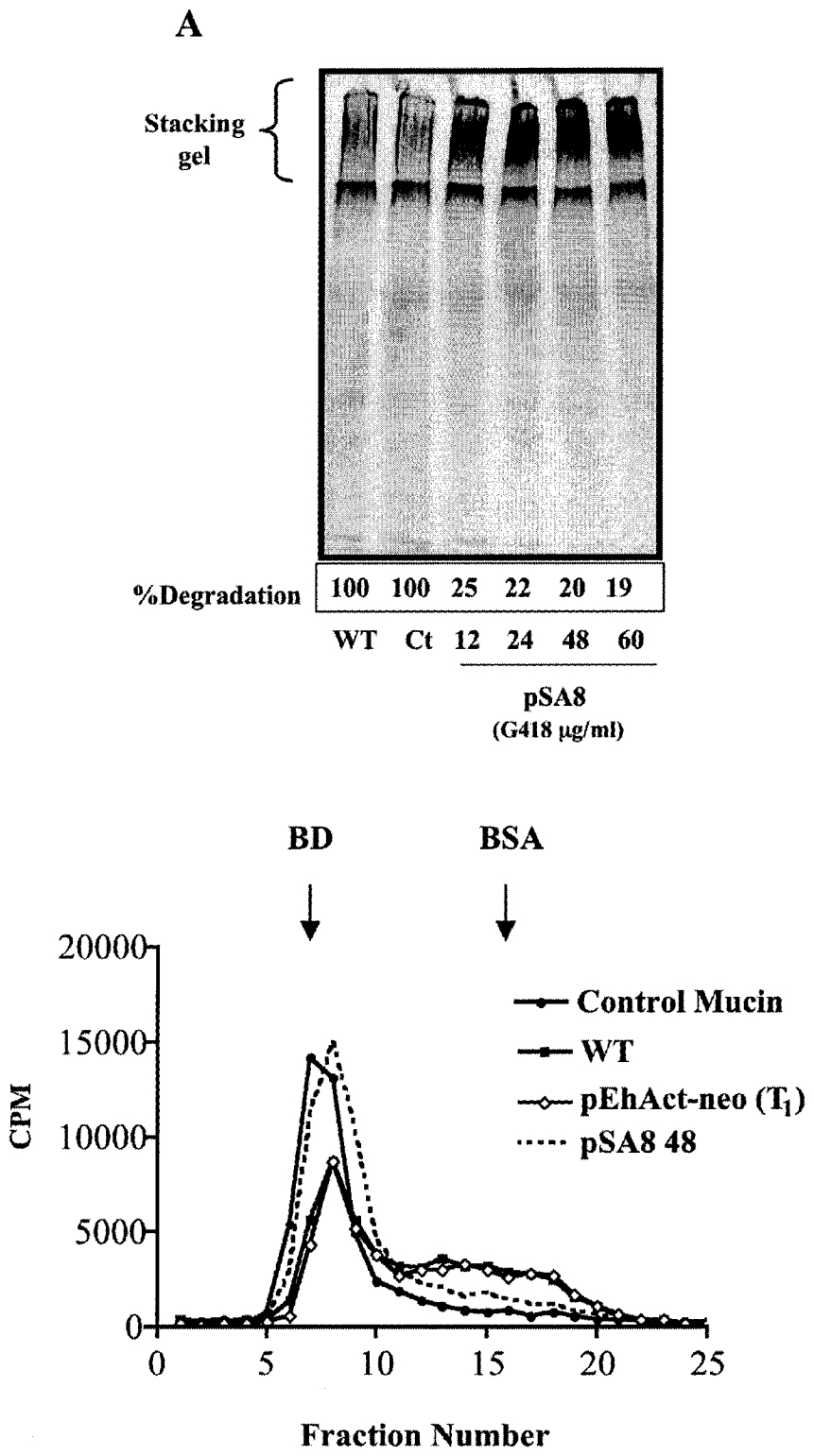
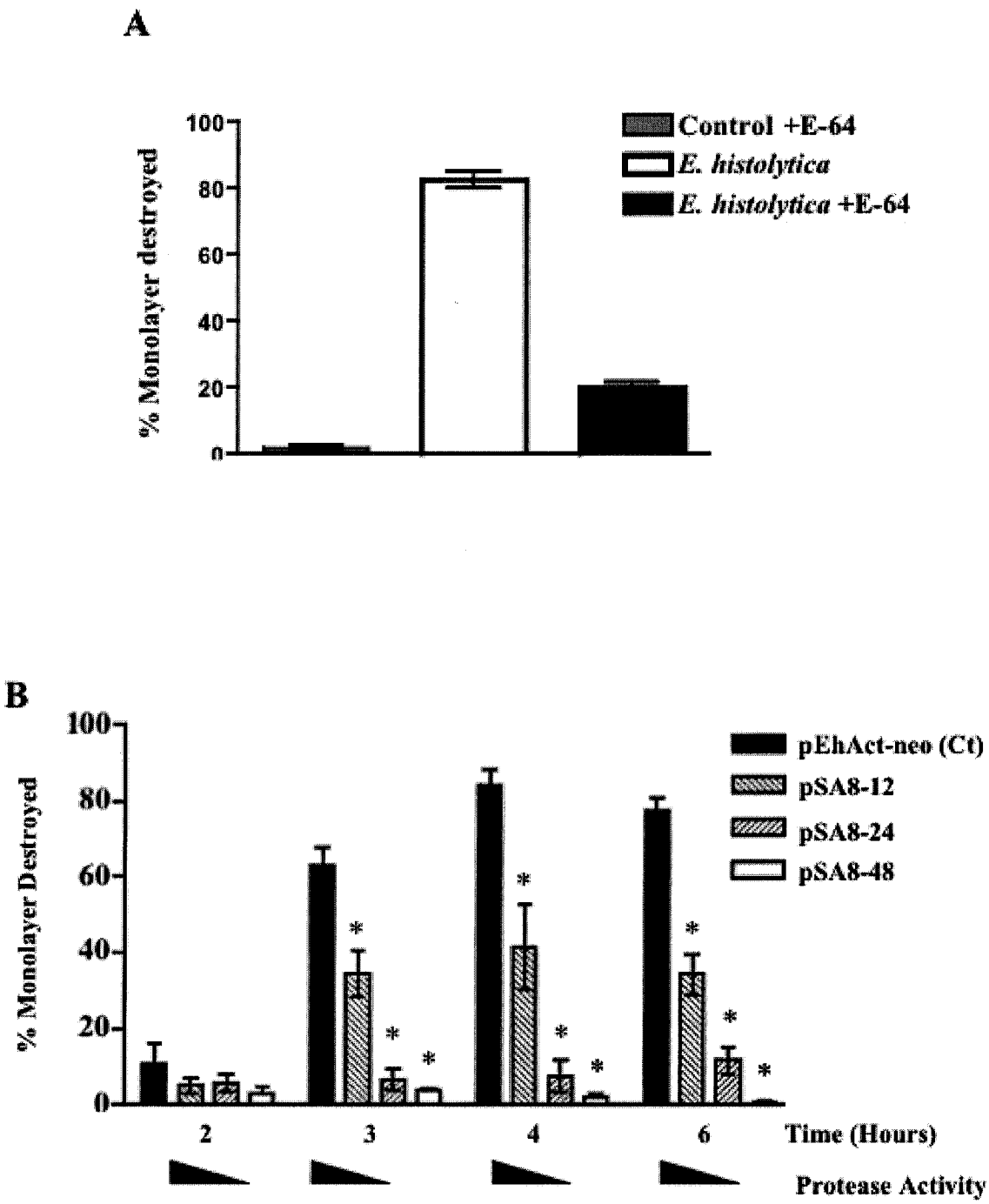


Figure 5.3. Mucin degradation assays of wild type and pSA8 transfected *E. histolytica.* (A) SDS-PAGE and autoradiograph of [^{35}S]cysteine labeled LS 174T mucin.

Radiolabeled mucin (2×10^4 cpm/digest) was incubated with 100 μg of *E. histolytica* cell lysate from wild type (WT), pEhActNeo grown in 12μg/ml of G418 (Ct) or pSA8 transfectants grown in increasing concentrations of G418 for six hrs at 37°C, separated by SDS-PAGE and visualized by autoradiography. (B) Digests were separated by gel filtration using a Sepharose 4B column. Mucin in the absence of *E. histolytica* lysate was used as a control. The column was calibrated with blue dextran (BD 2000 kDa) and bovine serum albumen (BSA 67 kDa, Amersham Biosciences, Uppsala, Sweden).

Cysteine Protease Activity is Required by *E. histolytica* to Destroy a Protective Mucus Barrier and Disrupt the Colonic Epithelium

Wild type trophozoites overcome a mucus barrier and destroy LS 174T cell monolayers by 80 ± 3% within three hours of incubation (**Fig. 5.4 A**). However, when wild type trophozoites were preincubated with the specific cysteine proteinase inhibitor E-64, it prevented disruption of the mucus barrier and subsequent monolayer destruction by 75 ± 5 %. These results suggest that cysteine proteinases are used by the parasite to disrupt the mucus gel. To address whether cysteine proteinase activity was important in overcoming the protective mucin barrier, we tested the ability of the pEhAct-neo and pSA8 strains of the parasite to invade colonic epithelial cells. As shown in **Fig. 5.4 B**, the pEhAct-neo strain (12 μg/ml G418) readily overcomes the mucus barrier and kills LS 174T cells in a time-dependent manner. In contrast, the pSA8 transfectants grown in the presence of increasing concentrations of G418 (12, 24, and 48 μg/ml) (pSA8-12, -24, -48) showed decreased protease activity and a corresponding decrease in monolayer destruction. In the CP deficient amoebae, only the pSA8-12 trophozoites were capable of causing a significant increase in monolayer destruction of 34% after 6 hrs incubation; this strain retains only ~ 45% of the cysteine proteinase activity of WT amoebae. The pSA8-24 and -48 transfectants with less than 20 and 10% of total cysteine protease activity, respectively, could not destroy an LS 174T monolayer with an intact mucus barrier after 6 hrs of incubation.



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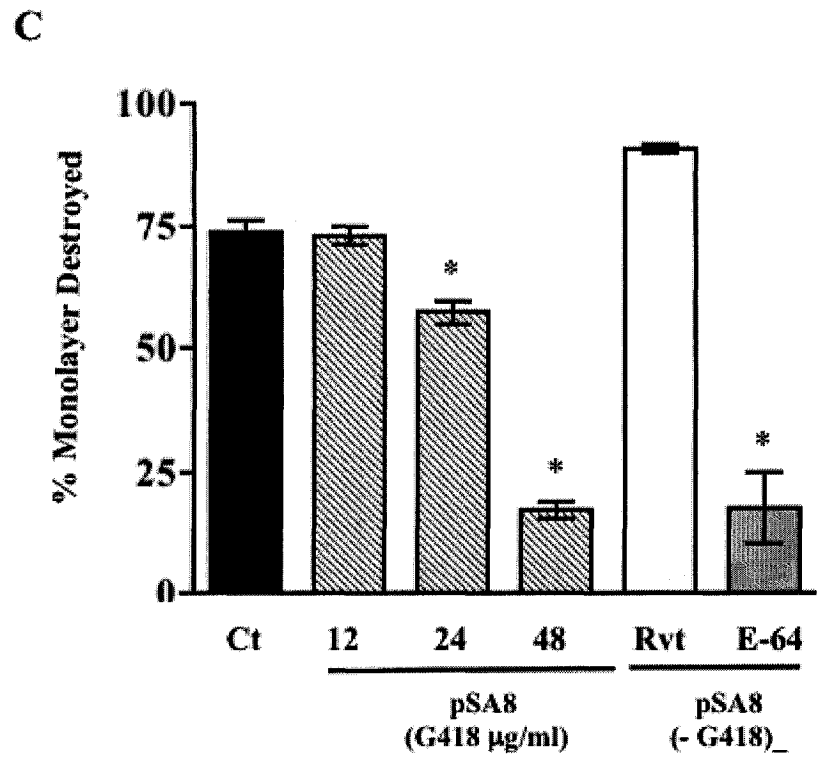


Figure 5.4. LS 174T cell monolayer destruction and mucin degradation by *E. histolytica* cysteine proteases. (A) Destruction of an LS 174T cell monolayer by *E. histolytica* trophozoites. Colonic cells were incubated with 100 µM E-64 (control), with *E. histolytica* trophozoites (1×10^5), or trophozoites and E-64 for 3 hours. (B) Destruction of an LS 174T cell monolayer by *E. histolytica* trophozoites. Mucin producing LS 174T cells were incubated with *E. histolytica* trophozoites transfected with the pEhAct-neo (12 µg/ml G418) or pSA8 plasmids grown in 12, 24, and 48 µg/ml of G418. (C) CHO cell monolayers, which do not produce gel-forming mucin were incubated for 3 hrs with the transfectants as well as the pSA8 48 revertant (Rvt) grown in the absence of G418 for three months or in the presence of 100 µM E-64 (E-64). For killing assays, the pSA8 transfectants were compared to the respective control (Ct) for each time point. * denotes a *P* value of < 0.05.

As cysteine proteinases are critically important in disrupting the mucus gel, we tested whether WT and CP deficient amoebae could equally destroy a CHO cell monolayer devoid of a protective mucus barrier. CHO cells are more susceptible to invasion than LS 174T cells since they do not secrete gel-forming mucins and were used to determine the actual contribution of the cysteine proteinases in mucus disruption versus epithelial cell invasion. As shown in **Fig. 5.4 C**, the pEhAct-neo strain destroyed 74% of a CHO monolayer, while the pSA8-12 destroyed 73% within three hrs. A 39% increase in CHO monolayer destruction was observed, compared to LS 174T monolayer invasion for the same time point. Although the pSA8-24 and -48 were unable to significantly destroy an LS 174T monolayer, both transfectants retained their ability to destroy a CHO monolayer (**Fig. 5.4 C**), with the pSA8-24 destroying 57% and the pSA8-48 destroying 18%, respectively. pSA8-48 revertants grown in the absence of G418 for three months completely regained their ability to destroy CHO cell monolayers similar to WT parasites. Interestingly, revertants incubated with E-64 destroyed the monolayer at a similar rate as the pSA8-48 strain. These results clearly indicate that cysteine proteases are needed for degradation of the mucus gel prior to epithelial cell invasion.

Adherence of the pSA8 Transfectants to CHO Cells

Amebic adherence to target cells via the Gal-lectin to galactose and N-acetyl-D-galactosamine residues is a prerequisite for epithelial cell cytolysis [24]. The Gal-lectin of *E. histolytica* binds with high affinity to colonic mucin in colonization and to epithelial cells during tissue invasion. In the pSA8 transfectants, it is possible that decreased cysteine proteinase production could affect Gal-lectin processing and surface expression of the Gal-lectin. To determine if the pSA8 transfectants were deficient in adherence capabilities mediated by the Gal-lectin, we assessed each transfectant's ability to adhere to target epithelial cells. In addition, the inhibition of adherence via the Gal-lectin was also demonstrated in the presence of galactose. As shown in **Fig. 5.5**, 70% of WT trophozoites formed positive CHO cell rosettes, which was inhibited by 50% in the presence of purified colonic mucin or 50 mM galactose. The pSA8-24 and -48 trasfectants only showed a slight decrease in CHO cell rosette formation compared to control transfectants. More importantly, the pSA8-48 amoebae formed CHO cell rosettes

that were inhibited by 34% in the presence of 50 mM galactose suggesting that the transfectants were not deficient in Gal-lectin binding capacity to mucin and to target epithelial cells.

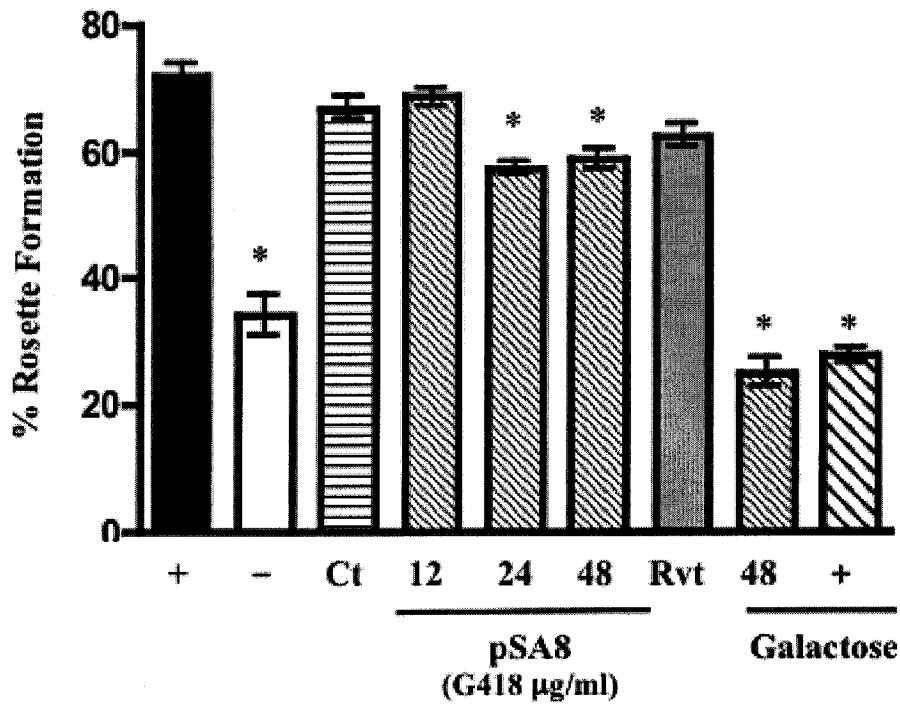
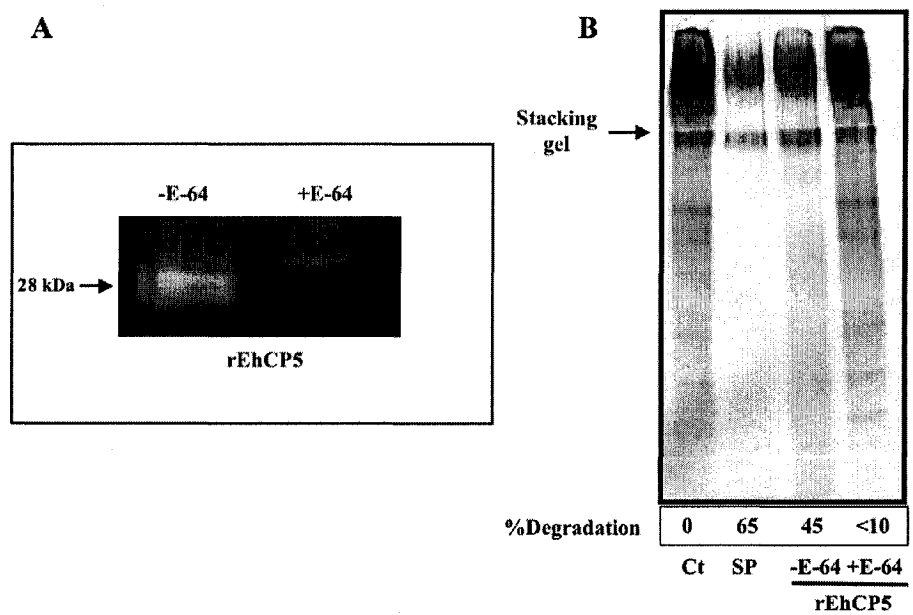


Figure 5.5. Adherence of *E. histolytica* transfectants to CHO cells. CHO cells were incubated with WT trophozoites (+) or with trophozoites preincubated with 1 µg of colonic mucin (-) for one hr. Adherence of pEhAct-neo (Ct), pSA8 transfectants and a pSA8-48 revertant (Rvt) was determined, as well as pSA8-48 and WT trophozoites in the presence of 50 mM galactose. An *E. histolytica* trophozoite adherent to three or more CHO cells was considered a positive rosette formation. Adherence of transfectants was compared to control (Ct), and adherence of trophozoites (WT) treated with mucin or galactose was compared to untreated trophozoites (solid black bar). * denotes a *P* value of < 0.05.

Recombinant EhCP5 Degrades Colonic Mucin

EhCP5 is one of the major surface associated and secreted cysteine proteinases produced by *E. histolytica*. The *EhCP5* gene is highly degenerated, and is not expressed in the noninvasive and closely related *E. dispar*; therefore it is hypothesized to be a likely virulence factor involved in host cell invasion [28]. The recombinant enzyme was expressed as an insoluble fusion protein in *E. coli*, and the refolding and activation of the enzyme was monitored by gelatin zymography (**Fig. 5.6 A**). Following activation, a 28 kDa band of activity was observed, and the enzyme was inhibited by E-64. The cysteine proteinase was then assessed for its ability to degrade [^{35}S]cysteine labeled mucin. As shown in **Fig. 5.6 B**, mucin was digested with *E. histolytica* secreted products as a positive control for degradation by cysteine proteinases, and 50 μg of secreted products degraded 65% of high molecular weight mucin. Similarly, the recombinant EhCP5 enzyme (4 μg) degraded 45% of high molecular weight mucin and was inhibited by E-64. Sepharose 4B gel filtration confirmed that the rEhCP5 degrades native mucin in a similar fashion as proteases secreted from live amoebae, demonstrating that EhCP5 is a major mucin-degrading enzyme (**Fig. 5.6 C**).



(Figure 5.6 C continued on page 124)

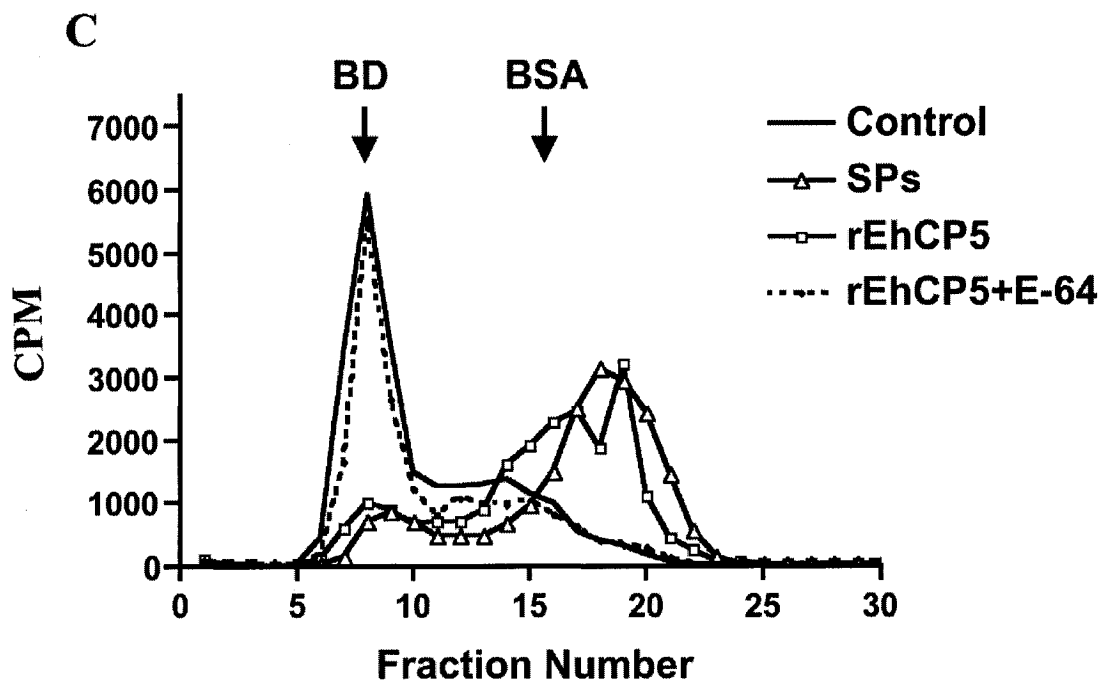
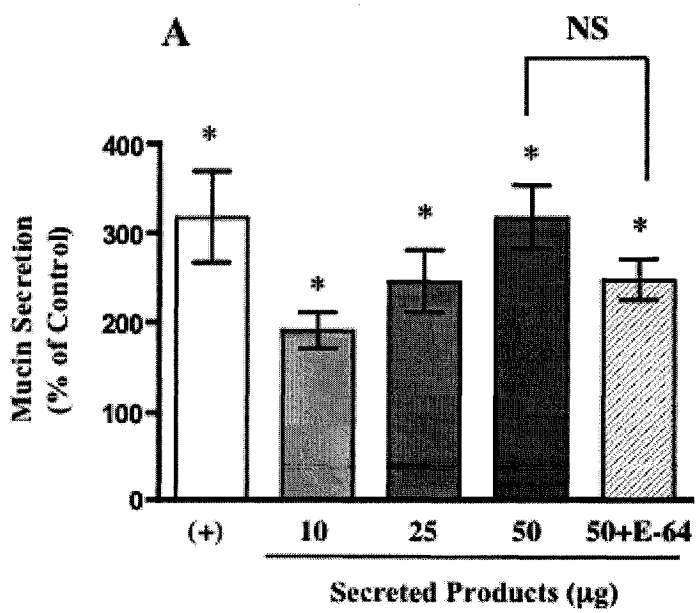


Figure 5.6. Degradation of colonic mucin by recombinant EhCP5. (A) Gelatin zymogram of activated recombinant EhCP5 in the absence or presence of E-64. Clear band indicates cysteine proteinase activity. (B) SDS-PAGE and autoradiograph of [^{35}S]cysteine labeled mucin digested with 50 μg of *E. histolytica* secreted products (SP), rEhCP5 (4μg) and rEhCP5 preincubated with E-64 (100 μM) for 6 h. The percent degradation was calculated as compared to the control mucin alone by densitometric analysis (Ct). (C) Sepharose 4B chromatography of [^{35}S]cysteine labeled mucin under the same conditions as (B).

E. histolytica Cysteine Proteinases do not Directly Evoke Mucin Secretion

To determine if *E. histolytica* cysteine proteases are involved in stimulating mucus secretion, amoeba secreted products as well as CP deficient amoebae were assayed for mucus secretagogue activity (**Fig. 5.7 A**). The secreted products induced mucin secretion in a dose-dependent manner with 25 and 50 μg inducing 245% and 317% secretion of the control, respectively. The addition of E-64 to 50 μg of secreted products had little effect on altering mucus secretagogue activity. These results were confirmed by Sepharose 4B column chromatography and the elution pattern of ^{3}H-labeled mucin demonstrated an increase in S4B V_0 material in response to amoeba-secreted components (data not shown). In a separate study, live trophozoites (2.5×10^5) induced low levels of mucin secretion during co-culture with epithelial cells using Millicell-HA membranes. There were no significant differences in mucus secretagogue activity between the pSA8-24 and -48 strains compared to the pEhAct-neo and WT trophozoites (**Fig. 5.7 B**). These results indicate that the mucus secretagogue released by the parasite is not a cysteine proteinase. Additionally, since mucus is continually secreted by the cells, and the cysteine proteinase deficient amoebae cannot digest the mucus, the trophozoites are impaired in their ability to traverse the mucus layer and are unable to reach the underlying epithelium to cause damage.



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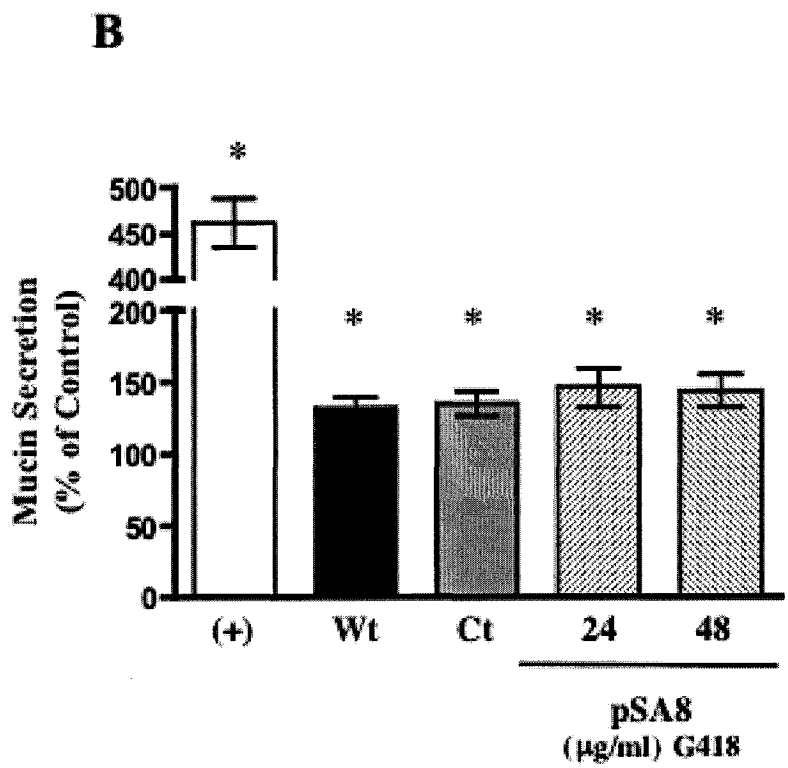

Figure 5.7. Secretion of ^{3}H-labeled mucin from LS 174T cells in response to *E. histolytica* secreted products. LS 174T colonic cells were incubated with 20 mM calcium ionophore A 23187 (+) as a positive control for mucus secretion, or various concentrations of secreted products, and secreted products with 100 μM E-64. Mucus secretion was assessed after 4 hrs incubation at 37°C. (NS) no significant difference between the two samples. (B) A co-culture system of LS 174T cells with WT trophozoites and transfectants was used to measure mucus secretion in response to live parasites. Mucus secretagogue activity was plotted as the percent secretion of control. The secretion in both experiments was compared to that of normal baseline secretion of the negative control (100 % secretion). * denotes a *P* value of < 0.05.

DISCUSSION

The present study provides evidence that *E. histolytica* cysteine proteinases are directly involved in the early events of invasive amebiasis. More specifically, the parasite causes a disruption of the innate mucus barrier. We have previously shown that *E. histolytica* secreted products from WT trophozoites degraded [^{35}S]cysteine labeled colonic mucin, which was inhibited by the cysteine protease inhibitor E-64. These results led us to investigate the specific contribution of the cysteine proteinases in mucus gel disruption and epithelial cell invasion.

An *in vitro* model of intestinal mucus penetration has been developed by our laboratory to determine the role of the mucus blanket in impeding epithelial cell invasion by *E. histolytica* trophozoites [16]. LS 174T cell monolayers produce large quantities of mucin and are currently the only in vitro model available for the study of invasive amebiasis prior to epithelial cell contact. Previously, we have shown that these cells are more resistant to destruction than CHO cells due to the protective mucus layer, in addition, inhibition of *O*-linked glycosylation with Benzyl-α-GalNAc in LS 174T cells facilitated rapid monolayer disruption by the parasite [16]. In the present study, the specific cysteine protease inhibitor E-64 prevented trophozoites from making contact with and destroying LS 174T monolayers and provided evidence for the involvement of CPs in mucus disruption.

Generation of cysteine proteinase deficient amoebae using antisense technology as well as the expression of recombinant cysteine protease 5 has made it possible to directly assess the contribution of the cysteine proteinases in invasive amebiasis [20]. *E. histolytica* trophozoites expressing the antisense message to *EhCP5* retain low levels of CP activity (<10%), and exhibit a loss of mucinase activity. Additionally, inhibition of CP activity directly influenced the parasite's ability to overcome a mucus gel and make contact with target epithelial cells, which resulted in a marked decrease in LS 174T monolayer destruction. Cysteine protease activity had a less significant effect on the ability of the parasite to disrupt a CHO cell monolayer lacking a mucus blanket. To

ensure that antisense inhibition of the CPs did not result in alterations of the Gal-lectin, and eliminate the possibility that the parasites were unable to adhere to epithelial cells, amebic adherence to CHO cells was assessed. Inhibition of protease activity had little effect on the ability of the parasite to bind to target cells and galactose specifically inhibited adherence of the pSA8-48 transfectants to CHO cells to the same extent as control amoebae. These results indicate that the pSA8 transfectants are not adherence deficient and their inability to invade an epithelial cell monolayer was not due to a lack of adherence to either mucin or epithelial cells. During its course of invasion, *E. histolytica* evokes a massive secretion of mucin from colonic cells [29]. It is hypothesized that this hypersecretion may cause an imbalance between mucus production, secretion, and degradation, resulting in a net reduction of the mucus blanket. This mucus depletion is thought to be one of the main factors contributing to epithelial cell invasion by the parasite. In our study, the cysteine proteases did not significantly contribute to mucus secretion as the pSA8 transfectants evoked mucus secretion to the same extent as wild type amoebae. These results confirm that additional parasite virulence factors involved in mucus depletion were unaffected by antisense expression.

E. histolytica secretes a small number of cysteine proteinases which may be involved in mucus disruption and cell invasion. These enzymes have similar substrate specificities and the proteases secreted by the parasite are all likely to independently degrade mucin and are not likely to work synergistically. Of the few cysteine proteinase genes that are expressed by the parasite, *EhCP5* is the only homologous gene not expressed by the non pathogen *E. dispar* due to the fact that the gene is highly degenerated, and this has raised interest in determining a role for this enzyme in mucin degradation and invasive amebiasis [26, 30]. EhCP5 is the only secreted cysteine proteinase known to re-associate with the parasite surface and exhibit increased activity when bound to membranes [31]. To date, attempts to over-express this enzyme in *E. histolytica* and *E. dispar* by plasmid transfection have been unsuccessful, making expression of an active recombinant EhCP5 enzyme a necessity to determine a function for the proteinase in invasion [22]. Until now, no specific protease has been identified as being directly involved in mucus degradation.

This study has defined a specific role for this group of proteinases and EhCP5 in particular, in overcoming the innate defense of the mucus layer.

Recent advances in the study of the synthesis and structure of MUC2 have increased our understanding of how the mucin polymeric network can maintain its integrity under the constant assault of pancreatic digestive enzymes. MUC2 is assembled into trimers and the folded protein contains a trypsin resistant trefoil domain [32]. These studies revealed the mechanism by which intestinal mucin is partially resistant to digestive enzymes such as trypsin, but much is still unknown with regards to how enteric pathogens breach the mucus barrier and how amoebae cysteine proteinases contribute to mucin destabilization. Future studies should focus on determining the target cleavage sites on MUC2 by the cysteine proteinases in order to understand how the parasite disrupts mucin polymerization. Perhaps *E. histolytica* uses a combination of glycosidase activity [33] in addition to proteases to destabalize the mucus gel. The parasite cysteine proteinases may have evolved in such a way as to specifically target the poorly glycosylated regions of MUC2 at sites that compromise mucin polymerization. The cysteine proteases and EhCP5 in particular, are attractive targets for the development of chemotherapeutic agents or vaccines against invasive amebiasis due to their central role in weakening the mucus gel and disrupting epithelial barrier function.

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Connecting Statement II

In Manuscript I, we demonstrated that cysteine proteinases are responsible for the major mucinase activity of *E. histolytica*. In Manuscript II, we have shown that *E. histolytica* requires the action of cysteine proteinases to overcome an intact mucus barrier and invade colonic epithelial cells. The exact mechanism by which the parasite overcomes the mucus gel still remained unknown. In Manuscript III, our aim was to identify the cleavage sites on MUC2 targeted by the parasite, to depolymerize the mucin network. This was performed using recombinant MUC2 N- and C-terminal proteins as these portions of the molecule are critically involved in mucin polymerization.

Chapter 6: Manuscript III

Entamoeba histolytica Cysteine Proteinases Degrade the C-Terminal Flanking Region of MUC2 and Destabilize the Mucin Polymer*

Moncada, D.M., Lidell, M., Hansson, G.C., and Chadee, K.

*Manuscript in preparation for submission

ABSTRACT

The protozoan parasite *Entamoeba histolytica* invades the colonic epithelium following disruption of the mucus layer by an unknown process. Herein, we investigated the mechanism by which amoeba cysteine proteinases disrupt the mucin polymeric network. *E. histolytica* secreted cysteine proteinases were assayed for their ability to degrade recombinant MUC2 protein segments involved in polymerization, and produced by expression in CHO-K1 cells to allow for proper assembly of the recombinant proteins. These proteins corresponded to the entire MUC2 N-terminal flanking region up to amino acid 1379 and the C-terminal protein segment containing the last 981 amino acids of MUC2. Interestingly, the amino terminus was discovered to be resistant to proteolytic degradation by *E. histolytica* cysteine proteinases whereas the MUC2 C-terminus was specifically targeted by the proteinases at two cleavage sites. A minor cleavage site was located within the cysteine-rich VWF like D-domain and would not compromise MUC2 polymerization. However, the major cleavage site located between the VNTR and the D4 domain could result in destabilization of the polymer. Specificity for this unique cleavage site was demonstrated through site-directed mutagenesis, which prevented the action of the cysteine proteinases. Furthermore, *E. histolytica* secreted proteinases were shown to disassemble insoluble MUC2 gel isolated from LS 174T cells. These results unravel a major role for *E. histolytica* cysteine proteinases in disrupting the polymerization of the mucus gel in the pathogenesis of intestinal amebiasis.

INTRODUCTION

MUC2 is the major gel-forming mucin secreted by goblet cells of the small and large intestines and is responsible for maintaining luminal barrier function by forming a protective gel, covering the epithelium. The mucus layer forms a physical barrier between contents of the lumen and the intestines and prevents the attachment of pathogens to enterocytes due to an abundance of carbohydrate receptors. Assembly of the MUC2 polymer is initiated by the formation of disulfide linked dimers through its COOH-terminal cysteine-rich domains in a similar fashion to PSM and VWF dimerization [1-3]. Multimerization takes place after transport of the dimer to the Golgi network by interchain disulfide bonding between N-termini as in the case of PSM [4, 5]. Studies in which the recombinant MUC2 N-terminus was expressed in CHO cells revealed that the N-termini form trimers that are held together by a trypsin resistant core creating a trefoil-like structure [4] and indicate that MUC2 does not form linear polymers but rather branched structures. The irregular repeat and variable number tandem repeat mucin domains of MUC2 are heavily glycosylated with *O*-linked oligosaccharides on serine and threonine residues. These highly glycosylated mucin domains are resistant to proteolytic attack due to steric hindrance, preventing access of the proteases to the protein core. The globular ends of the mucin protein are less glycosylated and the protein core is exposed, although intra- and intermolecular disulfide bonds formed within the N- termini enable the protein to maintain its polymeric structure following proteolytic digestion by trypsin [4]. Breakage of the mucin polymer by reduction and/or proteolytic degradation inhibits gel formation and compromises the protective function of the molecule [6, 7]. Mucin gel formation occurs as a result of polymerization as well as noncovalent interactions between polymers and factors that interrupt these events can compromise the mucus gel.

In order for enteric pathogens to invade and make contact with the colonic epithelium, they must overcome the protective mucus barrier. Microbes are theorized to accomplish this by a variety of mechanisms including proteolytic degradation of the mucin polymer, degradation of mucin oligosaccharides, and inducing hypersecretion of mucus, all contributing to mucus depletion and invasion [8-11]. The protozoan parasite *Entamoeba*

histolytica colonizes the mucus layer of the colon by adhering to mucin oligosaccharides via a 170 kDa Gal/GalNAc-adherence lectin [12]. In most cases of amebiasis, *E. histolytica* causes little harm to the host and remains in the mucus layer feeding on bacteria; however, in a small percent of infections, the parasite is able to overcome the mucus barrier and invade the underlying epithelium. We have previously shown that *E. histolytica* secretes cysteine proteinases, which degrade the cysteine-rich flanking regions of LS 174T cell colonic mucin. Moreover, the degraded mucin was less effective at inhibiting amebic adherence to target epithelial cells, indicating that the mucin polymer must be intact to maintain its protective function [6]. This same observation has been reported for *Candida albicans* when mucin was degraded by the secretory aspartyl proteinase Sap2p [13]. *E. histolytica* trophozoites expressing the antisense to *EhCP5* [14] have an impaired ability to disrupt an intact colonic mucus barrier and invade epithelial cell monolayers (Chapter V). These observations indicate that the parasite cysteine proteinases facilitate invasion of the colon by disrupting the innate defenses of the mucus gel. The exact mechanism by which the parasite compromises the mucin polymer has yet to be determined.

The aim of this study was to characterize the interactions between *E. histolytica* secreted cysteine proteinases and the N- and C- terminal flanking regions of MUC2 involved in polymerization. To achieve this, we expressed the N- and C- termini of MUC2 as recombinant proteins in CHO-K1 cells and examined the ability of the cysteine proteinases to degrade the secreted forms of the MUC2 ends. We found that while the N-terminus was completely resistant to proteolytic attack by *E. histolytica* proteinases, the C-terminus was cleaved at two distinct sites, which can depolymerize the MUC2 gel. This is the first study to identify a specific mechanism used by an enteric pathogen to disrupt the polymeric structure of mucin.

METHODS

Cell Culture and Preparation of *E. histolytica* Secretory Components

CHO-K1 [American Type Culture Collection] cells as well as the colon adenocarcinoma cell line LS 174T (ATCC) were cultured as described previously [15]. LS 174T (ATCC) cells were cultured in minimal essential medium (MEM) (Invitrogen) supplemented with 10% (v/v) fetal calf serum (Hyclone Laboratories), 100 μg/ml of streptomycin sulfate, 100 U of penicillin per ml, and 20 mM HEPES (Invitrogen) [16]. The CHO-K1 cells expressing the recombinant MUC2 N-terminal cysteine-rich domain were grown in serum free ProCHO3-CDM medium (Biowhittaker) [4].

E. histolytica HM1:IMSS trophozoites were serially passaged through gerbil livers to maintain high virulence and were cultured in TYI-S-33 as described previously [16]. Secretory components were collected from trophozoites incubated in Hank's balanced salt solution (Invitrogen) for two hours at 37°C at a final concentration of 2×10^7 amoebae/ml [17]. The cysteine proteinase activity in the secretory components was measured against the synthetic substrate z-Arg-Arg-pNA (Bachem) and assayed by gelatin zymogram [18]. The viability of the trophozoites was determined using the trypan blue exclusion assay.

Expression of Recombinant MUC2 N- and C- Termini

The CHO-K1 cells stably expressing the recombinant N- and C-terminal cysteine-rich domains of the human MUC2 mucin "respectively" have been described before [3, 19, 20]. CHO-K1 cells were transfected with the expression vector pSNMUC2-MG containing the first 4191 base pairs of the *MUC2* gene sequence after the native signal sequence (GeneBank™/EBI accession number L21998) [21]. CHO cells were also transfected with the pSMG-MUC2C plasmid containing bases 12622-15708 of the *MUC2* gene sequence as previously described [3]. The IRTT sequence in the pSMG-MUC2C vector (located at positions 4320 to 4323 in the MUC2 sequence [22]) was mutated to ADAA by site-directed mutagenesis (QuickChange™ site-directed mutagenesis kit; Stratagene), using the oligonucleotides 5′-CTCCACACCCAGCATCGCCGACG

CCGCCGGCCTGAGGCCCTACC-3′ and 5′-GGTAGGGCCTCAGGCCGGCGGCGT CGGCGATGCTGGGTGTGGAG-3′. The obtained plasmid, pSMG-MUC2C IRTT(4320-4323)ADAA was transfected into CHO-K1 cells using Lipofectamine 2000 (Invitrogen) and stable clones were selected and screened as described earlier [3]. Both constructs contained the gene encoding GFP and a MycTag. The MUC2 genes were ligated in frame with the GFP sequence and the immunoglobulin κ-chain signal sequence was used to direct the MUC2 termini into the secretory pathway [4].

Purification of Recombinant MUC2 Terminal Cysteine-rich Domains

Selection of CHO-K1 cells transfected with the pSNMUC2-MG, pSMG-MUC2C2, and pSMG-MUC2C2 IRTT(4320-4323)ADAA plasmids and detection of positive clones for expression was determined by G418 and fluorescence as previously described [3, 4]. Both the mutated and the non-mutated recombinant MUC2 C-terminal and MUC2 N-terminal cysteine-rich domains were purified as described previously for [3, 4]. Briefly, spent culture media were centrifuged at 1,000 × *g* for 10 min at 4°C and 0.02% (w/v) of NaN_3 was added. The samples were further purified by ultrafiltration and dialyzed against 50 mM Tris/HCl buffer (pH 8.0). The MUC2 C-terminal protein was further purified by ion exchange chromatography on a Mono Q column and by gel filtration on a Superose 6 HR column as previously described [3].

Antibodies and Affinity Purification of Rabbit Antiserum

The polyclonal antiserum, α-MUC2C2 directed against amino acids 4995 to 5013 (CIIKRPDNQHVILKPGDFK) located C-terminal to the D4 domain of the apoprotein was generated against synthetic peptides in New Zealand white rabbits as described previously [7]. The α-mycTag monoclonal antibody (MAb) was from spent culture media of the 1-9E10.2 hybridoma (ATCC, CRL-1729). Other antibodies used were Goat-anti-mouse immunoglobulins coupled to horseradish peroxidase (Goat-α-Mouse-HRP) (Pierce) and Goat-anti-rabbit immunoglobulins coupled to alkaline phosphatase (Goat-α-Rabbit-AP) (DAKO). Antibodies were purified from the serum by ammonium sulfate precipitation and protein G Sepharose chromatography according to the manufacture's instructions (Amersham Biosciences).

Purification of Insoluble Mucins from LS 174T Cells

LS 174T cells were cultured in 6-well plates for 10 days with daily media changes, and washed twice in cold PBS. The cells were extracted with guanidinium chloride (6 M guanidinium chloride, 5 mM EDTA, 10 mM NaH_2PO_4 [pH 6.5], 5 mM *N*-ethylmaleimide, and 1 mM phenylmethylsulfonyl fluoride), and incubated for 40 min at 4°C under agitation. Insoluble material was pelleted by centrifugation for 20 min at 30,000 × *g*, and the pellet was washed and centrifuged six times in similar guanidinium chloride buffer followed by similar washing six times in Dulbecco's phosphate buffered saline (DPBS) or 100 mM Tris-HCl [pH 8.5]. The resulting insoluble MUC2 gel was used for degradation studies.

Digestions with Amoeba Secretory Products

Amoeba secretory proteins were preincubated in DPBS with or without 100 μM E-64 (Roche) at 37°C for 30 min. Purified MUC2 C-terminal cysteine-rich domain or MUC2 N-terminal cysteine-rich domain from spent culture medium was then added to the mixtures. The recombinant MUC2 N-terminal fusion protein and the recombinant MUC2 C-terminus were incubated with DPBS alone or with 1 and 1.5 μg of *E. histolytica* secretory components and 1.5 μg pretreated with 100 μM E-64 (Roche) in DPBS for 6 hours at 37°C. The negative controls were incubated in DPBS only. The incubations were stopped by heating the samples for 5 min at 95°C.

When insoluble mucins from LS 174T cells were digested with Eh secreted proteins, the secreted proteins were pre-treated as above and transferred to insoluble mucins extracted from one of the wells of the 6-well plate. The mixtures were then incubated at 37°C for 16 hours. The negative controls were incubated in DPBS only. The incubations were stopped as above, centrifuged at 16,000 × *g* for 10 min and the resulting pellets were photographed.

SDS-polyacrylamide Gel Electrophoresis and Silver Staining

The samples were mixed with Laemmli sample buffer with or without 100 mM DTT, heated for 5 min at 95°C and analyzed by discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [23]. The Precision Protein Standards (Bio-Rad) were used as a molecular mass marker. Silver staining was performed according to the method described by Blum *et al.* [24].

Western Blot Analysis of Recombinant MUC2 Digests.

The recombinant MUC2 N- and C- terminal digests were analyzed by SDS-PAGE under native and reducing conditions using a 3-10% gradient gel with a 3% stacking gel as previously described [3]. After transfer of the proteins to PVDF membranes (Immobilon-P_{SQ}, Millipore), the membranes were placed in blocking solution (PBS containing 5% (w/v) milk powder, 0.1% (v/v) Tween-20 and 0.05% (w/v) NaN_3) overnight at 4°C and then incubated with either α-mycTag MAb (diluted 1:10) or α-MUC2C2 (diluted 1:100) for 2 h at room temperature. The membranes were washed 3 x 5 min with PBS-T (PBS containing 0.1% (v/v) Tween-20) and incubated with secondary antibodies (either Goat-α-Mouse-HRP 10 ng/ml or Goat-α-Rabbit-AP 1:1000 in blocking solution without NaN_3) for 1 h at room temperature. After another wash in PBS-T (3 x 5 min) the blots were developed using either the SuperSignal West Pico Chemiluminescent Substrate (Pierce) or NBT/BCIP (Promega).

Edman Sequencing of the Major MUC2 Cleavage Products

8.6 μg of recombinant MUC2 C-terminal cysteine-rich domain was digested with 6 μg of secreted proteins for 4 hours at 37°C and was separated by SDS-PAGE and blotted to a PVDF membrane as above. The membrane was stained with Coomassie blue and the stained bands were excised and N-terminally sequenced by Edman degradation on a Procise 492 Protein Sequencer (Applied Biosystems). A pulsed-liquid sequencing method for PVDF-blotted protein was used according to the manufacturer.

RESULTS

Expression of the Recombinant MUC2 N- and C- Terminal Proteins

To study the specific interactions between the *E. histolytica* cysteine proteinases and MUC2, it was necessary to express the regions of the molecule that are involved in polymerization, the MUC2 N- and C- termini. This was essential as the MUC2 polymer is extremely large (> 5 million Daltons) and is difficult to process in its native form [7, 25]. As shown in **Fig. 6.1 B**, the first 1397 amino acids of the MUC2 N-terminus were expressed as a fusion protein with a MycTag, followed by GFP. The protein was secreted from CHO cells as a trimer held together by intramolecular disulfide bonds between MUC2 N-termini [4]. In addition, the entire MUC2 C-terminus including amino acids 4198-5179 was also expressed as a fusion protein (**Fig. 6.1 C**). This recombinant protein was secreted by CHO-K1 cells as a dimer which was covalently joined by intramolecular disulfide bonds formed within the last 150 amino acids of the monomers [3]. The mutated MUC2 C-terminus was also expressed. The recombinant MUC2 proteins assemble in a similar fashion to native MUC2 termini, in addition, the secreted proteins are glycosylated similar to native MUC2 termini and were used for subsequent degradation studies.

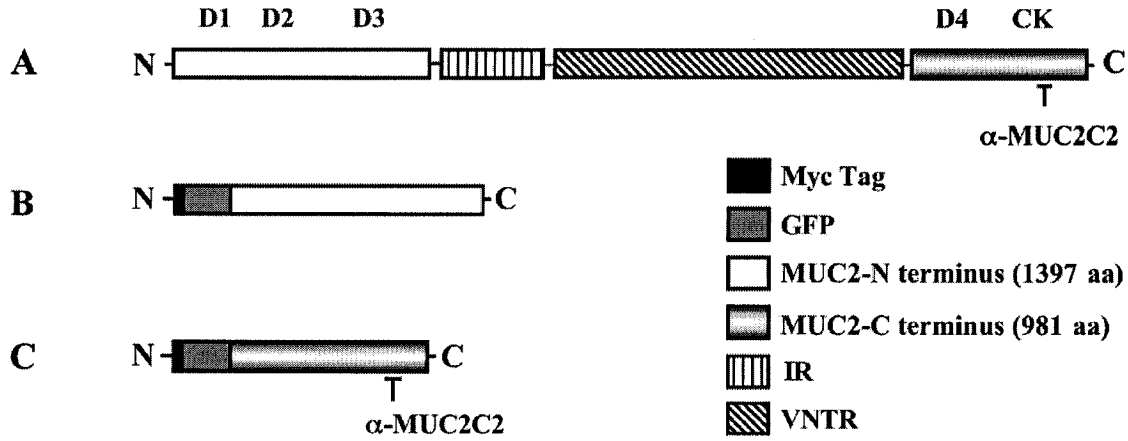


Figure 6.1. Schematic diagram of the MUC2 apoprotein and the recombinant MUC2 fusion proteins. (A) The entire protein sequence for MUC2 mucin. The sequence

includes the N-terminus, the irregular repeat (IR) mucin domain, the variable number tandem repeat (VNTR) and the C-terminus. VWF D-domains (D1-4) are located in the terminal flanking regions and the C-terminus contains a cysteine knot (CK) motif. (B) The coding region of the MUC2 N-terminus was expressed as a fusion protein with a MycTag and GFP in CHO-K1 cells. (C) The MUC2 C-terminus recombinant protein was expressed in CHO-K1 cells as in (B). The MUC2 C-terminus was also expressed with the amino acid substitutions from IRTT(4320-4323)→ADAA. α-MUC2C2 indicates an antibody directed against an epitope located C-terminal to the D4 domain.

Analysis of MUC2 Termini Degraded by *E. histolytica* Cysteine Proteinases

To determine the mechanism by which *E. histolytica* overcomes the protective mucus barrier, we digested the recombinant MUC2 N- and C-terminal proteins with amoebae secreted cysteine proteinases and analyzed the degradation products by silver staining. The MUC2 digests were initially separated under reducing conditions in order to visualize the monomeric forms of the proteins. As shown in **Fig. 6.2 A**, the MUC2 N-terminus incubated in DPBS alone as a negative control migrated on an SDS-PAGE gel with an apparent molecular mass of approximately 260 kDa, and was completely resistant to proteolytic degradation by amoebae proteinases. In contrast, however, *E. histolytica* secretory components markedly degraded the recombinant MUC2 C-terminus and generated two cleavage fragments, one with a molecular mass of 170 kDa and the second with a molecular mass of 75 kDa (**Fig. 6.2 B**). Moreover, pretreatment of the secretory components with the cysteine proteinase inhibitor E-64 specifically inhibited mucin degradation, and migration of the recombinant protein was identical to the monomeric form of the MUC2 C-terminus which migrated as a 250 kDa band due in part to the addition of glycans to the apoprotein [3]. Analysis of the undigested MUC2 C-terminus under non-reducing conditions yielded a band with a molecular mass of 470 kDa by silver staining (**Fig. 6.3 A**). Incubation of the dimer with amebae secretory components resulted in the liberation of a 300 kDa product and the degradation was also inhibited with E-64.

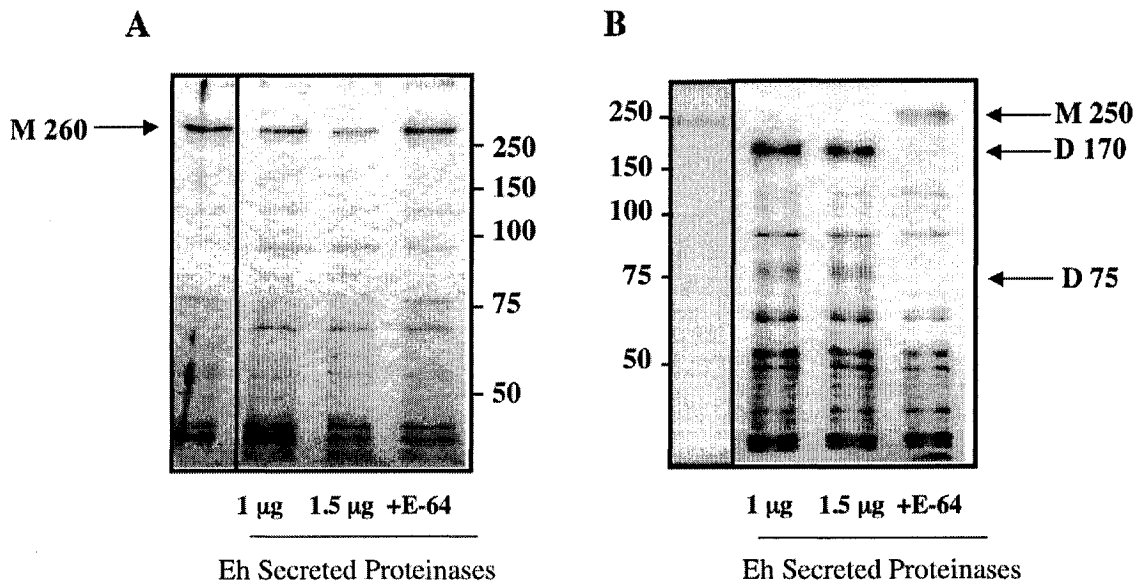


Figure 6.2. SDS-PAGE and silver staining of recombinant MUC2 proteins digested with *E. histolytica* secreted proteinases. The recombinant proteins were incubated with 1 and 1.5 µg of *E. histolytica* (Eh) secreted proteinases or 1.5µg secreted proteinases treated with E-64. (A) MUC2 N-terminus was separated using SDS-PAGE [3-10% gradient] under reducing conditions before and after exposure to Eh secreted proteinases. The monomer (M 260) has an apparent molecular mass of 260 kDa. (B) MUC2 C-terminus was incubated with the Eh secreted proteinases and analyzed as stated in (A). (D 170) MUC2 C-terminal cleavage fragment with a molecular mass of 170 kDa and (D 75) cleavage fragment of 75 kDa. Positions of the molecular weight markers are indicated to the right of the gel in (A) and to the left in (B).

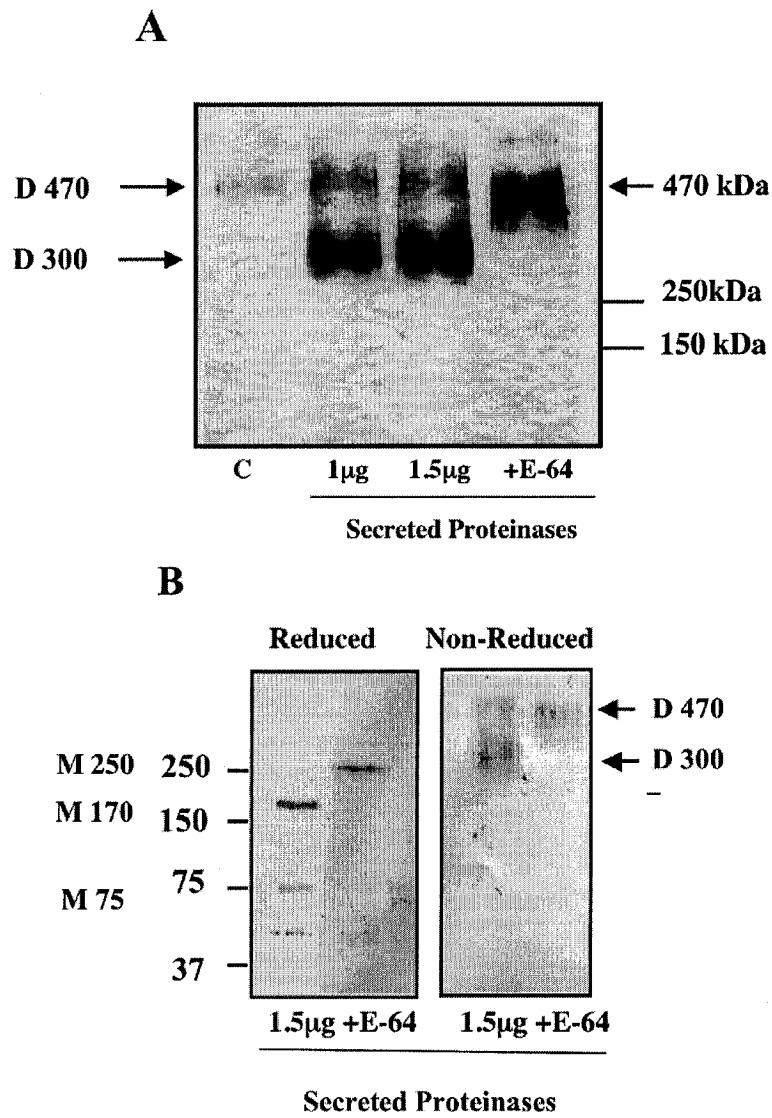
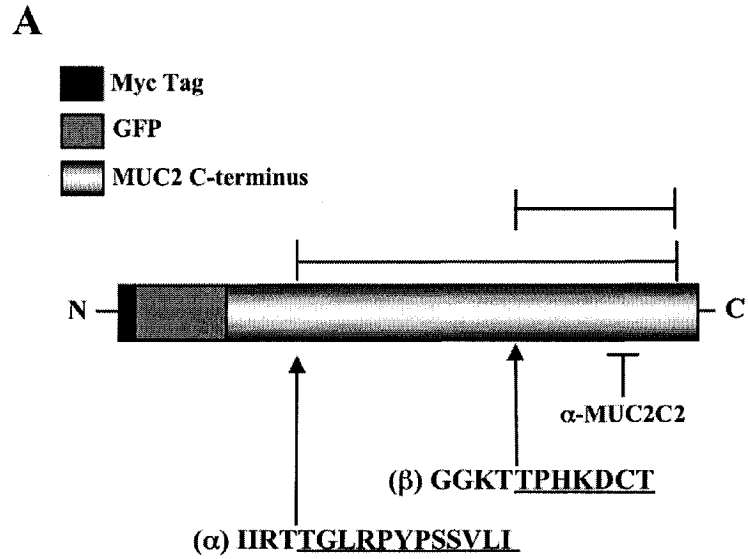


Figure 6.3. Characterization of the assembled MUC2 C-termini degraded by *E. histolytica* cysteine proteinases. (A) SDS-PAGE and silver staining of MUC2 C-terminus analyzed under non-reducing conditions. The 470 kDa homodimer of the MUC2 C-terminus (D 470) and the 300 kDa dimer (D 300) degraded by 1 and 1.5 μg of secreted proteinases or 1.5 μg secreted proteinases pretreated with E-64. (B) Western blot analysis of degraded MUC2 C-terminus separated by SDS-PAGE under either reducing or non-reducing conditions. The blots were probed with the α-MUC2C2 antibody. (M 250) non-digested MUC2 C-terminal monomer, (M 170) 170 kDa fragment, (M 75) 75 kDa fragment.

The identity of the degraded MUC2 C-terminus was investigated by western blotting and epitope mapping using an antibody directed against an epitope located within the last 150 amino acids of the protein known to be involved in polymerization (**Fig. 6.3 B**). The α-MUC2C2 antibody recognized both the 170 kDa and the 75 kDa fragments. A band of 250 kDa was also observed when the secreted products were pretreated with E-64. In addition, the antibody recognized the 300 kDa dimer indicating that the cleavage site(s) likely occurs outside this region. The α-MycTag antibody reacted with a fragment of ~30 kDa under non-reducing conditions (data not shown). These results demonstrate that *E. histolytica* cysteine proteinases specifically target the MUC2 C-terminus, and possibly cause breakage of the MUC2 polymer.

Identification of the Major Cleavage Sites on MUC2 by *E. histolytica* Cysteine Proteinases

N-terminal sequencing of the 170 kDa and 75 kDa bands shown in **Fig. 6.2 B**, was performed to delineate the locations of the cysteine proteinase cleavage sites. Sequencing of the peptides revealed that the cysteine proteinase activity was responsible for cleaving two regions of the protein resulting in major and minor cleavage sites (**Fig. 6.4 A**). The 75 kDa band was generated as a result of a minor cleavage, while the 170 kDa band was the major product released after treatment of the MUC2 C-terminus with amoebae secretory components. The peptide fragments were mapped to the protein sequence of MUC2 and found to be located within two distinct regions of the molecule (**Fig. 6.4 B**). The minor fragment was released after cleavage within the peptide sequence KT-TPHKDCT, and the major fragment was released after cleavage between the peptides RT-TGLRPYPSSVLI. *E. histolytica* cysteine proteinases have been reported to cleave substrates with arginine or lysine in the P_2 position [26] and the identified cleavage sites are in agreement with this specificity.



B

Domains

METDTLLLWVLLLWVPGSTGDAAQPARRAVRSSSELTSEQKLISEEDLSSATGATMVSKGEELFTGVVPILVELDG Igκ/Myc
DVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQ
ERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHN GFP
*IEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYK*SGLRSR
AQASNSAVDGTAGPGSTGSRVTHTSTAPIAELTTSNPPPESSTPQTSRSTSSPLTESTTLLSTLPPAIEMTSTA
PPSTPTAPTTTSGGHTLSPPPSTTTSPPGTPTRGTTGSSSAPTPSTVQTTTTSAWTPTPTPLSTPSI**IRTTGLR**
PYPSSVLICCVLNDTYYAPGEEVYNGTYGDTCYFVNCSLSCTLEFYNWSCPSTPSPTPTPSKSTPTPSKPSST
PSKPTPGTKPPECPDFDPPRQENETWWLCDCFMATCKYNNTVEIVKVECEPPPMPTCSNGLQPVRVEDP
DGCCWHWECDCYCTGWGDPHYVTFDGLYYSYQGNCTYVLVEEISPSVDNFGVYIDNYHCDPNDKVSCP
RTLIVRHETQEVLIKTVHMMPMQVQVQVNRQAVALPYKKYGLEVYQSGINYVVDIPELGVLVSYNGLSFS D4
VRLPYHRFGNNTKGQCGTCTNTTSDDCILPSGEIVSNCEAAADQWLVNDPSKPHCPHSSSTTKRPAVTVP
GGG**KTTPHKDCT**PSPLCQLIKDSLFAQCHALVPPQHYYDACVFDSCFMPGSSLECASLQAYAALCAQQN
ICLDWRNHTHGACLVECPSHREYQACGPAEEPTCKSSSSQQNNTVLVEGCFCPEGTMNYAPGFDVCVKT
CGCVGPDNVPREFGEHFEFDCKNCVCLEGGSGIICQPKRCSQKPVTHCVEDGTYLATEVNPADTCCNIT
VCKCNTSLCKEKPSVCPLGFEVKSKMVPGRCCPFYWCESKGVCVHGNAEYQPGSPVYSSKCQDCVCTD
KVDNNTLLNVIACTHVPCNTSCSPGFELMEAPGECCKKCEQTHCIIKRPDNQHVILKPGDFKSDPKNNC CK
TFFSCVKIHNQLISSVSNITCPNFDASICIPGSITFMPNGCCKTCTPRNETRVPCSTVPVTTEVSYAGCTKTV
LMNHCSGSCGTFVMYSAKAQALDHSCSCCKEEKTSQREVVLSCPNGGSLTHTYTHIESCQCQDTVCGLP
TGTSRRARRSPRHLGSG

Figure 6.4. Identification of the *E. histolytica* cleavage sites on the MUC2 C-terminus. (A) Edman sequencing of the 170 kDa and 75 kDa cleavage fragments revealed the N-terminal amino acid sequences of the peptides. Arrows indicate the site of cleavage and

underlined amino acid sequences indicate the N-terminal sequence of the (α) 170 kDa and (β) 75 kDa digestion products. (B) Schematic of the recombinant MUC2 C-terminal fusion protein indicating the cysteine proteinase susceptible cleavage sites. Amino acids are depicted as single letters and the sequences that are underlined and in bold are those identified by N-terminal sequencing of the cleavage fragments. The sequence underlined only, represents the epitope recognized by the α-MUC2C2 antibody. **C** indicates cysteine. The entire MUC2 C-terminal sequence begins following the italicized amino acid sequence of the GFP and MycTag fusion partners as well as the murine Igκ signal sequence. D4, Von Willebran factor like D4 domain. CK, cysteine knot motif.

The specificity of the cysteine proteinases for the major cleavage site releasing the 170 kDa band was demonstrated by mutating the cleavage site and changing the amino acid sequence from IRTT(4320)→ADAA. Replacement of the positively charged amino acid arginine with a negatively charged aspartic acid in the P_2 position of the peptide resulted in inhibition of degradation by the cysteine proteinases compared to the wild type MUC2 sequence (**Fig. 6.5 A**). This change in sequence would not prevent the cleavage of the minor site and could account for the appearance of two minor protein bands having molecular masses of ~175 and 75 kDa. Analysis of the digests containing the mutated protein under non-reducing conditions revealed that the dimer was intact after digestion with only a minor reduction in size after exposure to *E. histolytica* secreted proteinases (**Fig. 6.5 B**).

Degradation of the Insoluble MUC2 Complex by *E. histolytica*

Examination of the degraded mucin peptides clearly showed that the cysteine proteinases targeted two regions of the MUC2 C-terminus, but the functional significance of this event was not determined. To address this, insoluble MUC2 gel was extracted from LS 174T cells and incubated with various concentrations of *E. histolytica* secretory cysteine proteinases and examined for depolymerization of the mucin polymeric network. As shown in **Fig. 6.6**, a significant reduction in the insoluble mucin gel was observed following incubation with amoebae secretory components in a dose-dependent fashion. Moreover, mucin degradation was significantly inhibited by E-64.

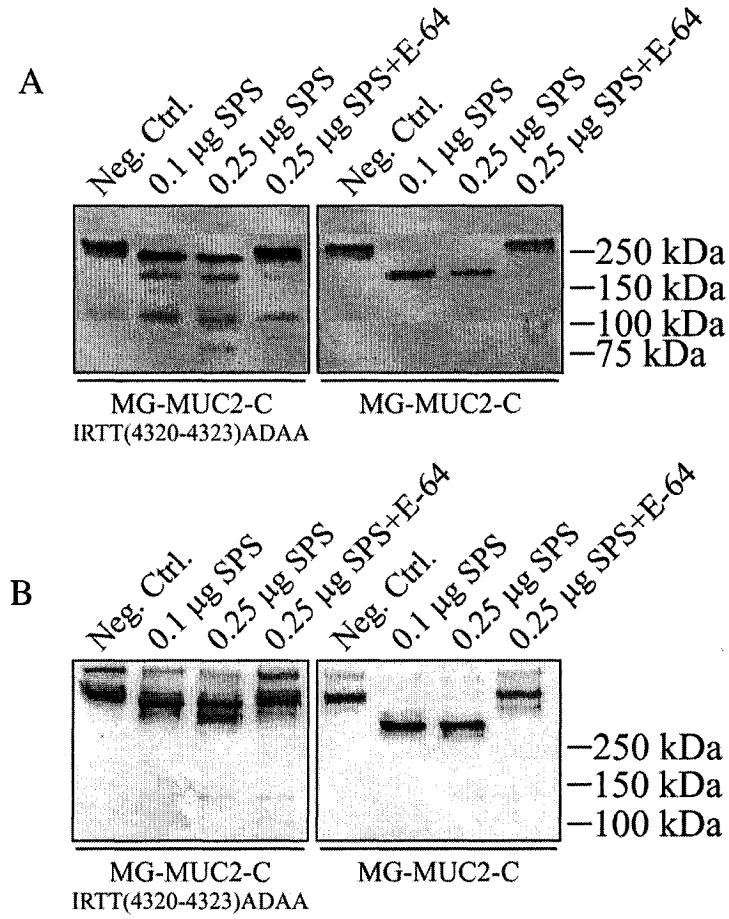


Figure 6.5. Digestion of mutated recombinant MUC2 by *E. histolytica* cysteine proteinases. Recombinant human MUC2 C-terminal cysteine-rich domain with the sequence IRTT (amino acids 4320 to 4323[22]) mutated to ADAA, was digested with either 0.1 or 0.25 μg of amoeba secretory products (SPs) for 3 hours at 37°C. Negative controls (Neg. Ctrl.) were treated with digestion buffer only and in the cases where the cysteine protease inhibitor, E-64, were used, the SPs were pretreated with the inhibitor for 30 min at 37°C before the recombinant mucin was added. The digests were separated on 3-10% SDS-PAGE gels under reducing (A) or non-reducing (B) conditions and the proteins were visualized by silver staining. MG-MUC2-C, recombinant MUC2 C-terminus; MG-MUC2-C IRTT(4320-4323)ADAA, mutated recombinant MUC2 C-terminus. Positions of molecular mass standards are indicated to the right.

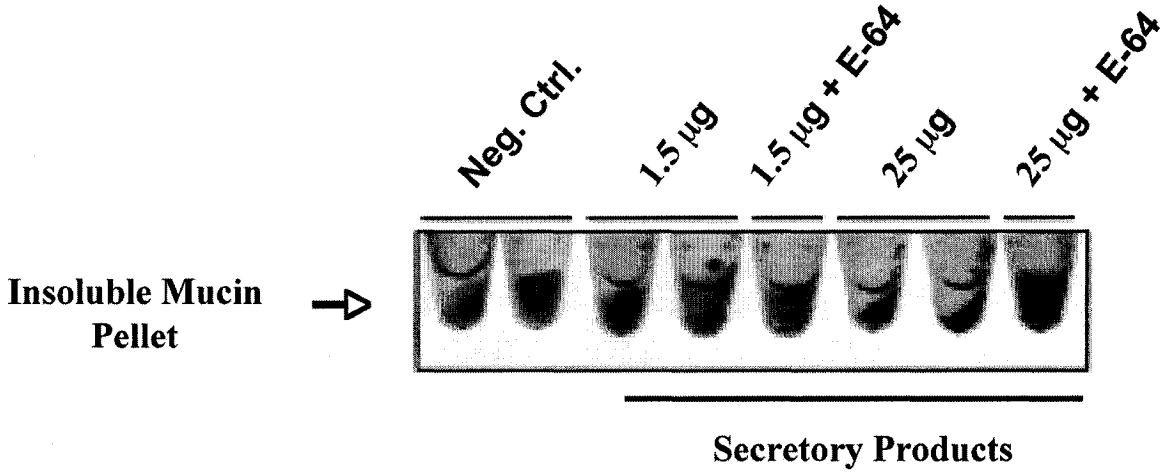


Figure. 6.6. LS 174T cells were extracted with guanidium chloride (6 M guanidium chloride, 5 mM EDTA, 10 mM NaH_2PO_4, [pH 6.5], 5 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride) and the remaining insoluble material, representing, the insoluble mucins, washed with Dulbecco's phosphate buffered saline (DPBS). The insoluble mucins were incubated with either 1.5 or 25 µg of amoeba secretory proteins (SPs) for 16 hours at 37°C. Negative controls (Neg. Ctrl.) were treated with DPBS only and in the cases were the cysteine protease inhibitor E-64 were used, the SPs were pretreated with the inhibitor for 30 min at 37°C before adding it to the insoluble mucin. Digestions were stopped by heating them to 95°C for 5 min. The samples were centrifued at 16, 000 x g for 10 min and the resulting pellets photographed.

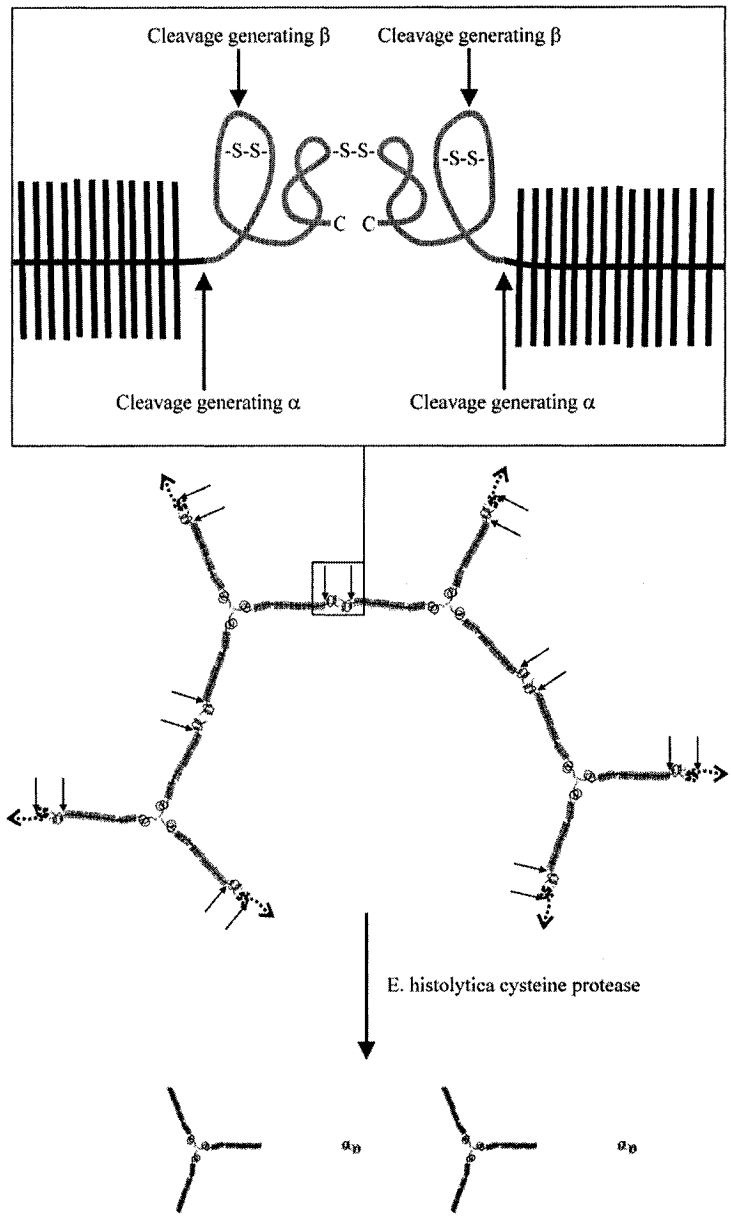


Figure 6.7. Cysteine proteinases secreted from *E. histolytica* depolymerize the MUC2 network. Cysteine proteinases secreted from *E. histolytica* cleave the MUC2 mucin at two positions in its C-terminal cysteine-rich domain generating the α and β fragments. While the mucus gel is still held together by disulfide bonding after the generation of the β fragment, the cleavage generating the α fragment disrupts the gel, thereby giving the amoeba access to the epithelial surface.

In order to predict if this degradation was sufficient to break the mucin polymer, it was necessary to examine the secondary structure of the C-terminal dimer (**Fig. 6.7**). The model predicts that the major cleavage resulting in the release of the 170 kDa band under reducing conditions, falls outside the areas involved in polymerization and is sufficient to break the mucin polymer. In contrast, the cleavage resulting in the β fragment would not be sufficient to disassemble the polymer due to intramolecular disulfide bridging enabling the molecule to maintain its structure.

DISCUSSION

Enteric pathogens must overcome a series of innate host defenses prior to making contact with the intestinal epithelium. The first obstacle encountered during invasion is the mucus barrier. Attachment to the host mucus layer and colonization of the gastrointestinal tract are the first steps in the infection process, which determine the outcome of disease. Tissue specific expression of mucin and mucin glycosylation patterns allow for the colonization of microbes in different regions of the gastrointestinal tract. For example, *Shigella dysenteriae* 1, the causative agent of shigellosis in humans, preferentially adheres to colonic mucin but not to small intestinal mucin [27]. *E. histolytica* colonizes the mucus layer of the colon by binding with high affinity to Gal and GalNAc residues of colonic mucin [12, 28]. The parasite also binds host epithelial cells via the Gal-lectin and this adherence is a prerequisite for epithelial cell cytolysis and invasion [28]. Mucin carbohydrates act as receptors for commensal gut microflora as well as invasive organisms and binding sites on mucins compete with those on the underlying epithelium and contribute to restricting pathogens access to the mucosa. In addition, mucus plays a protective role by entrapping pathogens resulting in expulsion with mucus flow during defecation [29].

Even after successful colonization of the gastrointestinal tract, invading bacteria, viruses, or parasites must overcome the mucus barrier. Virulence factors such as proteases, glycosidases, and mucus secretagogues are produced by these organisms and are responsible for disruption of the mucus gel. *E. histolytica* constitutively secretes at least

four cysteine proteinases into its environment that are involved in tissue destruction and invasive disease [18, 30]. The closely related non-invasive species *E. dispar* exhibits significantly less cysteine proteinase activity than *E. histolytica,* and does not express some homologous cysteine proteinases to *E. histolytica* shown to be involved in mucus degradation (Chapter V) [31, 32]. It is possible that high levels of cysteine proteinase activity in *E. histolytica* contribute to increased virulence and invasion. Previous studies have indicated that this activity plays a major role in intestinal as well as extraintestinal disease. Cysteine proteinase activity is necessary for the parasite to cause liver abscess [33, 34], migrate through host tissue [35], and disrupt epithelial cell monolayers in vitro [14]. In addition, cysteine proteinase activity was found to be essential for trophozoites to traverse a colonic mucus barrier prior to cell cytolysis [6] (Chapter V). The mechanism by which the parasite disrupts the mucin polymeric network was unknown, but there was evidence to support the idea that amoebae disrupt the cysteine-rich domains of MUC2 involved in polymerization [6].

In this study, we have identified the regions of the MUC2 polymer targeted by the *E. histolytica* cysteine proteinases. The molecular weights of MUC2 polymers cannot be assessed with accuracy by SDS-PAGE analysis due to the fact that the glycoproteins do not enter the gels under non-reducing conditions. This has made studies concerning the effects of enteric pathogens on intestinal mucins a challenge. Therefore, a more practical approach was taken to investigate the interactions of the *E. histolytica* cysteine proteinases with the MUC2 N- and C- termini. This was achieved by expressing the entire MUC2 N- and C- termini as recombinant proteins in CHO-K1 cells. The proteins were directed to the secretory pathway using the murine Igκ-chain signal sequence to ensure proper assembly and secretion of the recombinant proteins. Previous studies were conducted using these same expression systems in order determine how the MUC2 polymer is assembled and secreted [3, 4]. The MUC2 C-terminus was shown to form heterodimers, while the N-terminus was shown to be secreted as a trimer; this was clearly demonstrated by SDS-PAGE analysis and electron microscopy of the recombinant proteins [3, 4]. Using this system, we identified two cleavage sites on the MUC2 protein in the C-terminus targeted by the parasite. Both sites were in agreement with the

specificity of the proteases for arginine or lysine in the P_2 position of the peptide and sequencing of the two cleavage products identified their location to be at opposite ends of the C-terminus [36, 37]. Interestingly, non-reducing SDS-PAGE analysis revealed that even after digestion by the cysteine proteinases, the C-terminal dimer was still held together but was of an apparent lower molecular weight. This indicated that only one of the cleavage sites could be responsible for breaking the mucin polymer and this was located near the N-terminus of the recombinant protein between the VNTR and the D4 domain (see **Fig. 6.4**). These results are consistent with the intra and inter-molecular disulfide bonding occurring within the later portion of the C-terminus, which would allow the dimer to stay intact even after proteolytic degradation. Site directed mutagenesis of the main cleavage site was performed to demonstrate the specificity of the proteinases. Replacement of arginine with aspartic acid inhibited the majority of the cleavage and altered the degradation pattern of the recombinant protein, although the minor cleavage site was still targeted.

The mucus layer presents an obstacle for all invasive pathogens of the gastrointestinal tract that must be disrupted prior to contact with the epithelium. *E. histolytica* has developed a unique strategy to overcome the innate defense of the mucus barrier. In addition to degrading the mucin oligosaccharide component of the molecule [38] the parasite specifically targets the regions of the molecule involved in polymerization by proteolytic degradation. This event has not yet been documented for an enteric microorganism and may aid in our understanding of how invading pathogens defeat the innate defense of the mucus layer.

Support

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Connecting Statement III

In the preceding three manuscripts we demonstrated: 1) that *E. histolytica* degrades colonic mucin, 2) the parasite uses the action of cysteine proteinases to overcome a mucus barrier, and 3) that the parasite cysteine proteinases target the C-terminus of MUC2 and depolymerize the mucin network. We successfully identified the virulence factor involved in the initiating events of invasive amebiasis. Our studies focused on the effect of the parasite proteases on the mucin protein core, and not the oligosaccharide component of the molecule. Since many of the properties of the mucus gel are directly attributed to the oligosaccharide component of mucin, it was important to determine if *E. histolytica* glycosidases are also involved in mucus degradation. In Manuscript IV, we characterize the *E. histolytica* glycosidase activity against colonic mucin oligosaccharides.

Chapter 7: Manuscript IV

Entamoeba histolytica Secreted Products Degrade Colonic Mucin Oligosaccharides*

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ABSTRACT

Degradation of the mucus layer by *Entamoeba histolytica* is a prerequisite for invasion of the colonic mucosa. In this study we demonstrate that amoebae secreted products degrade ^{3}H-labeled and native colonic mucin oligosaccharides independent of proteolytic activity. We conclude that *E. histolytica* degrades mucin oligosaccharides, which may facilitate parasite invasion of the colon.

Entamoeba histolytica is responsible for at least 50 million cases of diarrhea and an estimated 100,000 deaths per annum and ranks second only to malaria as a cause of mortality due to a protozoan parasite [1]. Infection with the parasite leads to amebic colitis and colonic ulceration and less frequently, dissemination to the liver resulting in amebic liver abscess. The initial events leading to invasion of the colon by *E. histolytica* are poorly understood and the mechanisms used by the parasite to overcome the innate host defenses of the gastrointestinal tract are currently under investigation. The parasite colonizes the colonic mucus layer by binding mucin oligosaccharides via a 170 kDa Gal/GalNAc lectin and must traverse this protective barrier in order to cause epithelial cell damage and colonic ulceration. Mucin oligosaccharides serve to protect the mucin core from proteases, preserving the integrity of the mucin polymer. Various *O*-linked glycan structures are attached to the apomucin via *O*-glycosidic linkage to serine and threonine residues and these *O*-glycan branches contain N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), fucose, galactose, and sialic acid. The oligosaccharide component of gastrointestinal mucin has been reported to account for up to 90% of its dry weight, and the densely packed oligosaccharides are responsible for many intrinsic physical properties of the mucus gel ranging from hydration, gel-forming capacity, protease resistance, and rigidity [2]. Previous studies have identified numerous glycosidase activities in *E. histolytica* lysates and secretory products. More specifically, the parasite has been found to produce a sialidase, an α-glucosidase, as well as β-N-acetylhexosaminidase, enzymes which are released by the parasite and are hypothesized to be involved in amebic pathogenesis [3-5]. These glycosidases may play a role in disrupting mucin by exposing the protein backbone to parasite proteases. Previously we have shown that *E. histolytica* secreted cysteine proteases degrade the poorly

glycosylated regions of MUC2 and we hypothesize that the parasite may use the concerted actions of glycosidases and proteases to disassemble the mucin polymeric network [6].

In the present study, we determined whether *E. histolytica* secreted glycosidases could degrade colonic mucin oligosaccharides. Parasite secretory products were collected from trophozoites incubated in HBSS for 2 hours and > 95% of trophozoites were viable as determined by trypan blue exclusion assay [7]. Secreted products were assayed for activity against a panel of glycosidase substrates as previously described with some modifications [8]. Briefly, 20 μg of secreted components (representing ~2 × 10^5 trophozoites) were assayed for glycosidase activities between pH 3.5 and pH 8.5 to determine optimal activity using various *p*-nitrophenyl (pNP) glycoside substrates (2mM) (EMD Biosciences Inc., San Diego, CA). One unit of enzyme activity was defined as the number of micromoles of substrate digested per minute per milligram of protein, and one unit of activity was considered significant.

Highly purified ^{3}H-labeled mucin as well as native mucin was collected from LS 174T colonic cells (American Type Culture Collection, Rockville, MD.) grown to 80% confluence in minimal essential medium (MEM) (Invitrogen Corporation, Burlington, Ontario, Canada) and purified by Sepharose 4B (S4B) gel filtration and/or cesium chloride density gradient centrifugation (CsCl mucin) as previously described [6, 9]. Mucin oligosaccharide degradation was assessed with native mucin and was visualized by Periodic Acid-Schiff, in-gel staining of the mucin oligosaccharides using the GelCode glycoprotein staining kit according to the manufacturer's instructions (Pierce, Rockford, IL). Western blot analysis was performed using an antibody generated in New Zealand white rabbits against LS 174T cell mucin which was purified by gel filtration and density gradient centrifugation [9]. Specificity of the antibody for mucin oligosaccharides was determined by oxidizing the mucin with 10 mM sodium metaperiodate (Sigma-Aldrich, Burlington, Ontario Canada) in phosphate buffered saline (Invitrogen Corporation) in the dark for one hour [10]. In addition, degradation of ^{3}H-labeled mucin glycoproteins was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE),

and fluorography as well as Sepharose 4B size exclusion chromatography, as previously described [6]. Secreted products were pretreated with protease inhibitors or with the complete-mini EDTA-free protease inhibitor cocktail according to the manufacturer's instructions (Roche GmbH, Mannheim, Germany). Trypsin and papain were used as a control for proteolytic degradation in the absence of glycosidase activity (Roche GmbH).

E. histolytica secreted products were found to contain abundant amounts of activity against various glycoside substrates (**Table 7.1**). The highest level of activity detected was that of β-*N*-acetyl-D-glucosaminidase at pH 7.0. In addition, high levels of α-D-glucosidase activities were also detected with maximal activity between pH 6.0 and pH 8.0 which is in agreement with a previous report [11]. Modest levels of β-D-galactosidase, β-L-fucosidase as well as α-N-acetyl-D-galactosaminidase were also detected.

TABLE 7.1. Glycosidase activity present in *E. histolytica* secreted products

Substrate	Activity (U)	Significance
β-N-Acetyl-D-Glucosamine	62	S
α-D-Glucose	46	S
β-D-Galactose	3.6	S
β-L-Fucose	3.2	S
α-N-Acetyl-D-Galactosamine	1.2	S
α-L-Fucose	-	NS
α-D-Mannose	-	NS
α-D-Galactose	-	NS

S, significant. NS, not significant.

Based on the various structures of human intestinal mucin oligosaccharides, all of these enzymes would be required to break down mucin oligosaccharides by the parasite [12, 13]. There has been no evidence to date that defines a role for these enzymes in mucin degradation, and previous methods used to examine oligosaccharide degradation by the parasite may have not been sensitive enough to detect minor changes in the structure of mucin due to its high molecular weight and polymeric nature. As shown in **Fig. 7.1 A**, *E. histolytica* secreted products degraded mucin and mucin oligosaccharides in a dose-dependent manner as evidenced by the migration of PAS reactive material into an SDS-PAGE running gel. Incubation of the mucin with as little as 10 μg of secreted products resulted in an 87% decrease in high molecular weight stacking gel mucin, while trypsin did not alter the migration compared to control mucin. Since cysteine proteases are the major class of enzyme released by the parasite, and have been shown to degrade the poorly glycosylated flanking regions of mucin [6], the involvement of these proteases in altering mucin oligosaccharide migration was assessed. Degradation of the mucin oligosaccharides by the parasite was not inhibited by the cysteine protease inhibitor E-64 or by the serine protease inhibitor Pefabloc-SC (**Fig. 7.1 B**). These results are of particular interest since E-64 has been shown to markedly inhibit the majority of proteolytic degradation of purified mucin by amoebae [6]. Treatment of the secreted products with a protease inhibitor cocktail was also ineffective at inhibiting the liberation of mucin oligosaccharides into the running gel (data not shown). Western blot analysis of the digests with an antibody that recognizes purified colonic mucin oligosaccharides showed a 56% reduction in immunoreactive mucin remaining in the stacking gel (10μg secreted products), while trypsin digestion of the mucin did not result in any significant loss of mucin carbohydrates from the stacking gel (**Fig. 7.1 C**). The α-mucin antibody did not recognize mucin in which the sugars have been oxidized, indicating that the antibody specifically recognizes mucin oligosaccharides (**Fig. 7.1 D**).

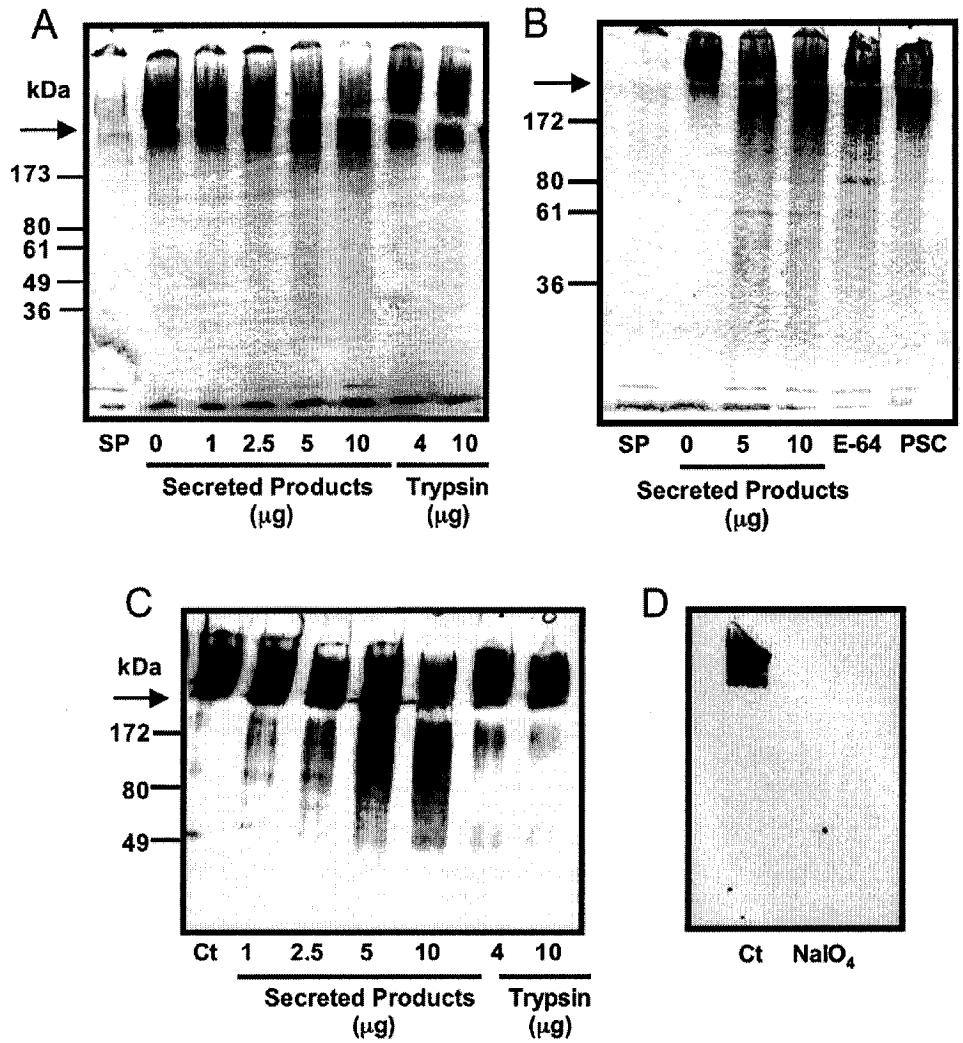

Figure. 7.1. PAS staining of native mucin treated with *E. histolytica* secreted products. (A) Dose-dependent degradation of CsCl purified mucin visualized by SDS-PAGE and PAS staining. (B) *E. histolytica* secreted components (10μg) were preincubated with E-64 and Pefabloc-SC prior to the digest. (C) Western blot analysis of mucin digests with a α-LS 174T cell mucin antibody. (Ct) Control. Arrow indicates border between stacking and running gel. (D) Western blot analysis showing specificity of the antibody for mucin oligosaccharides. (Ct) control mucin; $NaIO_4$, sodium metaperiodate treatment.

To address specific cleavage of mucin oligosaccharides, the mucin was metabolically labeled by incubating LS 174T cells with [^{3}H]glucosamine. Under these conditions, the glucosamine is converted to various carbohydrates and is incorporated into mucin glycoproteins. This allows for the tracking of the sugars and is useful for detecting minor alterations in mucin structure. As shown in **Fig. 7.2 A**, ^{3}H-labeled S4B V_0 (Void volume) mucin was degraded by amoebae secreted products in a dose-dependent fashion which was specifically inhibited by boiling. Amoebae glycosidase activity of ^{3}H-labeled mucin was similar to the degradation pattern observed with native colonic mucin (**Fig. 7.1 A**). More importantly, the addition of a variety of protease inhibitors had no effect on ^{3}H-labeled mucin degradation (**Fig. 7.2 B**), clearly demonstrating glycosidase activity. This method is more sensitive than PAS staining for detecting oligosacchride degradation and confirmed that the mucin carbohydrates were being disrupted. As shown in **Fig. 7.3 A**, ^{3}H-labeled mucin purified by CsCl density gradient centrifugation elutes in the V_0 of a S4B column. However, following incubation with amoebae secreted products there was a 51% decrease in void volume (fractions 6-11) mucin, and an increase in ^{3}H-labeled material in the included fractions (fractions 12-25). Although the size of the mucin molecule does change dramatically upon degradation with amoebae-secreted products, changes in the buoyant density of the molecule are not as dramatic, but are evidenced (**Fig. 7.3 B and C**). The degraded mucin does exhibit a wider range of densities and can be detected in fractions 5 to 7. Furthermore, mucin appeared to be solubilized by the secreted products, as evident by an increase in the total amount of recoverable ^{3}H-labeled mucin in the presence of secreted products as compared to controls (**Fig. 7.3 B**).

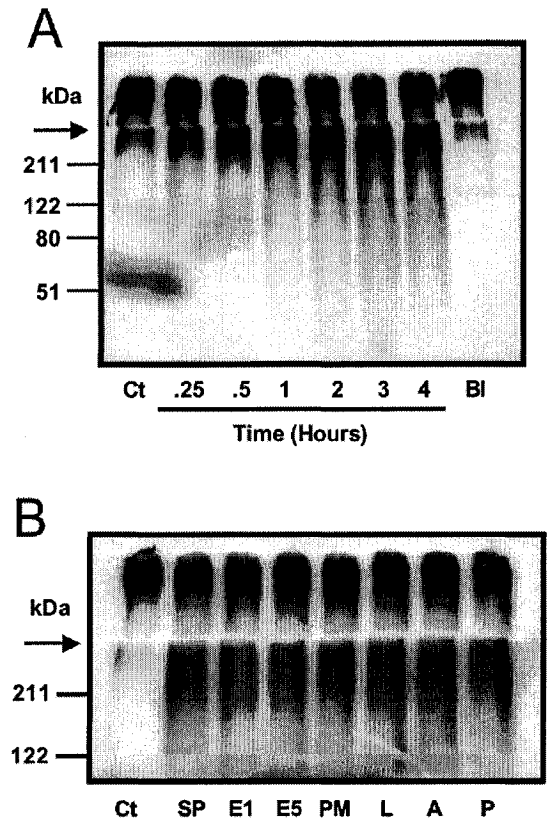

Figure 7.2. SDS-PAGE and autoradiograph of ^{3}H-labeled mucin degraded by amoebae secreted products. (A) Time-dependent degradation of ^{3}H-labeled mucin. Sepharose 4B purified mucin was incubated with 50 μg of amoebae-secreted products for up to 4 hours. Control (Ct). Mucin was also incubated with secreted products that were inactivated by boiling (Bl). (B) Effect of protease inhibitors on the degradation of [^{3}H]glucosamine labeled mucins. Secreted products (SP) were treated with the following protease inhibitors prior to incubation with mucin: E1 and E5 (E-64, 100 μM and 500 μM), PM (PMSF, 10 mM), L (leupeptin, 10 mM), A (aprotinin, 10 mM), and P (pepstatin, 10 mM) (Roche GmbH). The digests were performed for 6 hours. Arrow indicates border between stacking and running gel.

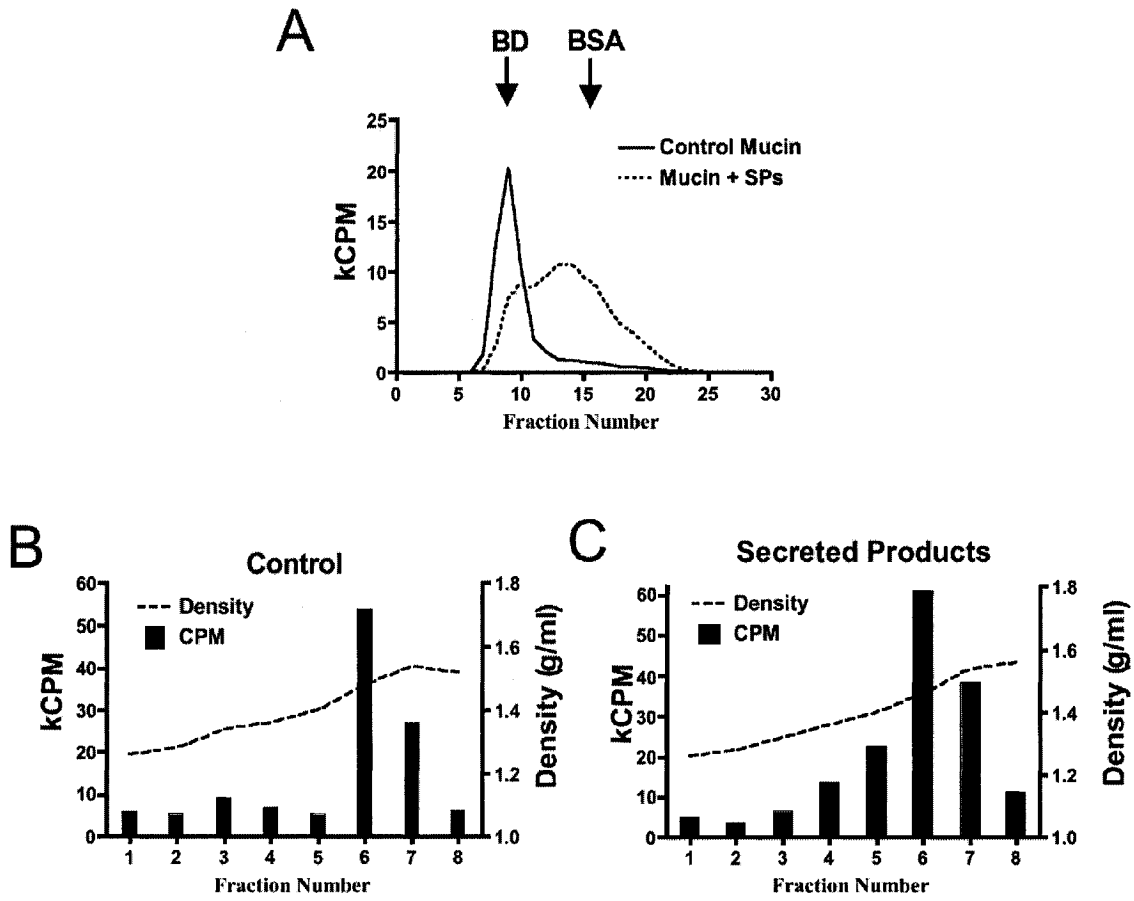

Fig. 7.3. Sepharose 4B gel filtration of ^{3}H-labeled CsCl mucin degraded by *E. histolytica* secreted products. (A) Purified mucin (3×10^{5} cpm) was incubated in PBS alone or with 200 μg of secreted products at 37°C for 18 hours. The digests were separated by gel filtration and aliquots of each fraction were analyzed by liquid scintillation counting. The column was calibrated with blue dextran (BD, 2,000 kDa, Pharmacia, Uppsala, Sweden) and BSA (Bovine serum albumin, 68 kD, Sigma-Aldrich). (B-C) CsCl density gradient centrifugation of ^{3}H-labeled mucin incubated in PBS (B) or with amoebae secreted components (C). Data represent the results of one experiment repeated two times with similar results.

We have previously demonstrated that *E. histolytica* cysteine proteinases are responsible for degrading the cysteine-rich regions of colonic mucin, and the degraded mucin is less effective at inhibiting amebic adherence to target Chinese hamster ovary cells [6]. Degradation of mucin by *E. histolytica* is likely to result in destabilization of the mucin polymer, solation of mucus, and subsequent loss of the protective function of the mucus gel. Although mucin polymerization is an important and essential element required for its gel formation, mucin *O*-linked oligosaccharides are necessary for protecting the protein backbone, including the mucin domains, from degradation by proteases. Although some previous studies have determined that the parasite does not produce the proper range of glycosidases to degrade mucin, we have found that the parasite does produce a wide range of glycosidases and the products secreted by the parasite are capable of degrading colonic mucin. This indicates that the parasite uses both protease and glycosidase activity to disrupt the mucin polymeric network. The liver fluke *Fasciola hepatica* also secretes glycosidases into its environment and the parasite's excretory-secretory products have been shown to degrade ovine mucin even in the absence of proteolyic activity [14]. Our results indicate for the first time that *E. histolytica* releases abundant amounts of glycosidases into its environment, and this activity is sufficient to degrade colonic mucin oligosaccharides. In addition, the ability of these enzymes to degrade mucin suggests they play an important role in allowing the parasite to overcome the innate defense of the mucus barrier.

SUPPORT

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Section III: General Discussion

Discussion

The epithelium of the gastrointestinal tract is continually exposed to chemical and physical insults as well as potential enteric pathogens. The mucus layer is a dynamic barrier in a constant state of turnover rather than a static state and is continually renewed by secretions from goblet cells. The normal gastrointestinal microflora also play a beneficial role in regulating mucus thickness by degrading mucins and utilizing them as a source of energy. A fine balance between mucin production and secretion must be maintained during these conditions in order to ensure adequate protection of the epithelium. One can think of the innate defenses of the GI tract as a series of obstacles and attacks that an "enemy" microbe must overcome to defeat the host. The offending microbe is itself armed with a battery of "weapons" to defeat these attacks. The mucus barrier acts as a nonspecific obstacle to invasion by physically trapping microorganisms and impeding the diffusion of microbial toxins, denying them access to the underlying epithelium. The host responds to pathogens by hyper secreting mucus, which is often sufficient for removal of the irritant. In addition, the mucus layer contains high levels of secretory IgA [1], lysozyme [1], antimicrobial peptides (defensins) [2] and trefoil factor proteins [3] to help combat the deleterious effects of microbes. All of these aforementioned factors contribute to the innate defense of the gastrointestinal tract. Trefoil factors in particular are known to be involved in epithelial restitution and have been shown to increase the viscosity of mucus [4] which may also contribute to stabilizing the interactions between mucin polymers.

In the event that microbes or their toxins breach the mucus barrier, the next line of innate defense comes into play. Epithelial cells form a physical barrier, keeping luminal contents from entering the body. These cells are joined together by tight junctions, which function as the "glue" between the cells and are composed of membrane spanning proteins present on the apical surface of the cells. Tight junctions act as a gate, restricting the movement of molecules between epithelial cells. In addition, they also serve a fence function by separating the apical and basolateral membrane components to maintain cell polarity [5]. Many invasive organisms have evolved mechanisms of altering epithelial

permeability by regulating or disrupting tight junctions. Finally, epithelial cells also express "microbial sensors" called Toll-like receptors. These receptors recognize pathogen-associated molecular patterns and allow the host to detect antigens on pathogens and mount an appropriate response to eliminate the offending organism (reviewed in [6]). Regardless of a strong innate host defense mechanism, in some instances, microbial pathogens invade the mucosal surface and cause disease. In the end, severity of disease is dependent upon the virulence of the organism as well as the host defense.

Clearly, maintenance of a functionally intact mucus barrier is the first action required by the host to protect the epithelium. MUC2, the major gel forming mucin of the colon forms long branched structures that assemble into a mesh-like polymeric network. Upon secretion, the heavily glycosylated polymers form a slimy visco-elastic gel in part because the oligosaccharide components become highly hydrated and swollen. In addition, mucin forms large aggregates in solution as a result of polymer cross-linking [7]. This noncovalent cross-linking between the mucin polymers is essential to gel formation. Mucin gel formation is reversed or inhibited when the cysteine-rich flanking regions are disrupted by either disulfide bond reduction or proteolytic degradation. Mucins may have evolved to withstand degradation by gastrointestinal proteases by containing cysteine-rich regions involved in polymerization that take on secondary structures such as trefoil like domains. These regions in the N-terminus of MUC2 enable the molecules to maintain their polymeric nature, even when cleaved, due to intramolecular disulfide bonding keeping the molecules intact [8]. Gastrointestinal pathogens must accordingly develop strategies to overcome these structural features. The heavily glycosylated irregular repeat and variable number tandem repeat regions of MUC2 are very heavily glycosylated and the oligosaccharides confer protease resistance to these domains. The glycosylation also causes the mucin molecule to take on an extended conformation while the poorly-glycosylated regions contain globular conformations. Intestinal microflora as well as pathogens have been shown to produce a range of glycosidases that are involved in mucin oligosaccharide degradation. In order for invading microorganisms to reach the intestinal epithelium, they must traverse the mucus

barrier. This may be accomplished by at least one, or a combination of the following events; 1) proteolytic degradation of the poorly glycosylated regions of mucin and breaking of the mucin polymer, 2) degradation of mucin oligosaccharides leading to exposure of the mucin apoprotein to degradation, and 3) hypersecretion of mucus in such a fashion that mucus secretion rates exceed mucin production rates, resulting in a net decrease in mucus gel thickness. Demonstrating these events in vivo would prove quite difficult since the host itself produces proteases and the commensal gut microflora contribute to mucin degradation. Additionally, other factors such as mechanical shearing of the loosely adherent mucus layer by intestinal contents and removal of mucus during peristalsis add more variables that would be difficult to distinguish from pathogen effects. Our lab has developed an *in vitro* model to study the interactions between enteric microorganisms and colonic mucin using the mucus producing colonic adenocarcinoma cell line LS 174T [9]. Previously, we have characterized the mucins secreted from these cells and have demonstrated that they are similar in composition to the major colonic mucin species of the human colon. LS 174T cells contain a mucus barrier and are more resistant to invasion by *E. histolytica* due to the protective effect of the mucus blanket, while non-mucus secreting cells are more susceptible to invasion. This system has allowed us to decipher the mechanisms used by *E. histolytica* to overcome the mucus barrier, and has made it possible to investigate the effects major parasite virulence factors have on mucin structure and function.

E. histolytica produces many virulence factors involved in the pathogenesis of invasive amebiasis but only a small number would likely be involved in mucin gel destabilization. The primary molecules interacting with the mucus layer are the Gal-lectin and cysteine proteinases. The Gal-lectin allows amebae to colonize the colon by binding to mucin oligosaccharides. This is the first key step in invasive disease but this event also occurs during noninvasive disease and does not actually contribute to alterations in mucin. Other virulence factors such as the cysteine proteinases are more likely to be key molecules involved in invasion since they have been shown to degrade a range of host proteins. Therefore, we hypothesized that *E. histolytica* cysteine proteinases degrade colonic mucin and facilitate invasion of the colon. We set out to test this hypothesis (manuscript

I), and our goal was to determine if parasite secretory products could degrade colonic mucin and if so, what class of protease is responsible for this event. This aim was accomplished by metabolically labeling LS 174T cell mucin with [^{35}S]cysteine to track the poorly glycosylated regions of mucin. Subsequently, mucin degradation assays were performed with Eh secretory components in the presence and absence of a variety of protease inhibitors. Cysteine proteinases were identified as the major mucinase activity by inhibition with the specific inhibitor E-64 [10]. Measuring the ability of the degraded mucin to inhibit adherence of the parasite to target epithelial cells allowed us to determine the functional significance of this degradation. Our results clearly showed that native mucin significantly inhibited adherence of the parasite to epithelial cells to a greater extent than degraded mucin, indicating that the polymeric nature of mucin must be maintained to confer adequate protection.

After discovering that the cysteine proteinases degrade the flanking regions of mucin, our next aim was to determine the role these enzymes play in traversing the mucus barrier and in epithelial cell invasion. This was achieved (in manuscript II) by generating cysteine proteinase deficient parasites through antisense technology. This strategy allowed us to investigate the contribution that the proteinases play in facilitating invasion of colonic epithelial cells. Using these transfectants in combination with our LS 174T cell colonic model, we demonstrated that the proteinase activity was necessary for the parasite to overcome a mucus barrier and destroy the underlying epithelium. This was achieved by comparing the ability of the parasite to destroy intact epithelial cell monolayers that secrete gel-forming mucins (LS 174T) or those devoid of a mucus barrier (CHO). The results observed between the two conditions were dramatic and the essential role for these proteinases in facilitating invasion of the mucus barrier was revealed. To address which of the proteinases may be involved in mucin degradation and mucus gel disruption we chose to express recombinant cysteine proteinase 5 in a bacterial expression system. This cysteine proteinase is of particular interest since it 1) is secreted by the parasite, 2) associates with the parasite membrane and exhibits increased activity in association with membranes and, 3) is not expressed in the non invasive commensal *E. dispar*. EhCP5 was found to be highly mucolytic and degraded mucin in a similar fashion

as total secretory proteinases. These results define a clear role for EhCP5 in invasive amebiasis as a virulence factor responsible for disruption of the colonic mucus layer.

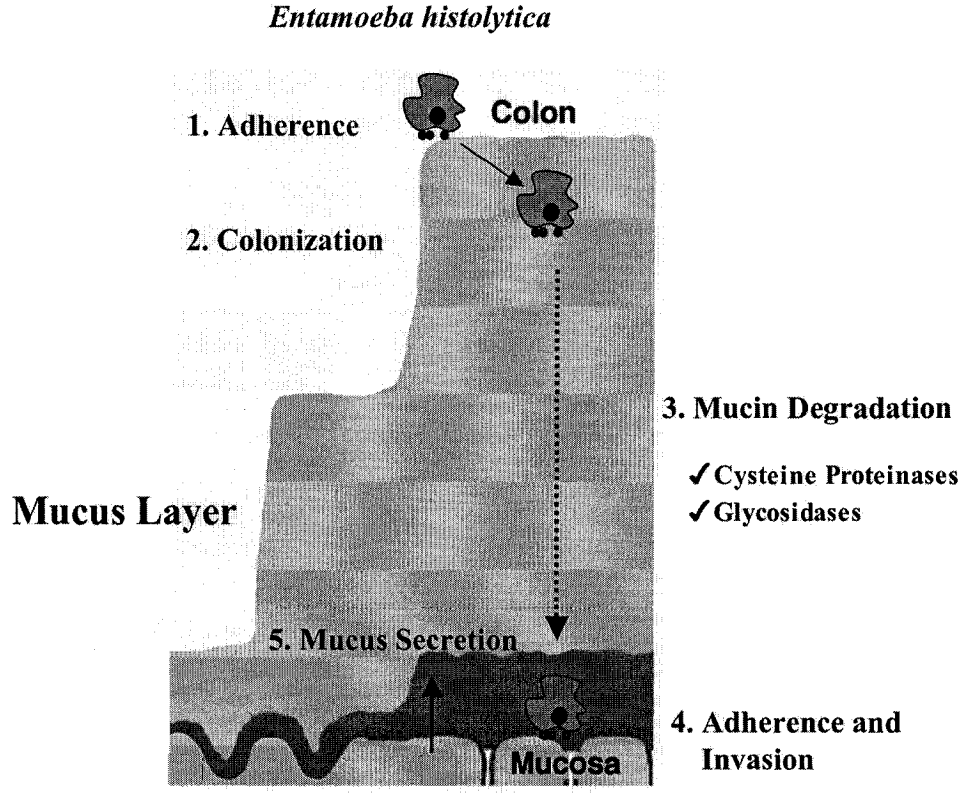
After discovering the major virulence factor responsible for allowing the parasite to overcome the innate defense of the mucus barrier, we sought to determine specifically how these enzymes are capable of disrupting the mucin polymeric network (manuscript III). Since mucin polymers are extremely large and virtually impossible to work with, we decided to direct our focus on the portions of the molecule involved in polymerization, the N- and C-terminal cysteine rich regions. This was accomplished by obtaining recombinant MUC2 C-terminal dimers and N-terminal trimers secreted from CHO cells. These recombinant proteins are held together by disulfide bonds in the same manner as those of native MUC2 polymers and enabled us to determine how EhCPs compromise these structures. Although the MUC2 N-terminus was resistant to degradation by the proteinases, we identified two cleavage sites on the MUC2 C-terminus, one major and one minor. Of particular importance, the major cleavage site falls outside the cysteine knot motif of the molecule and would allow for disassembly of the dimer. This major finding is the first reported mechanism by which an enteric pathogen destabilizes the mucin network.

Cysteine proteinases clearly have a destructive effect on the mucus barrier but the parasite produces other virulence factors that may be involved in mucin degradation. One characteristic of mucin is its abundant glycosylation. The oligosaccharides protect the protein core from damage by blocking proteases from coming in close proximity to the peptide. Interestingly, the parasite secretes a range of glycosidases that would be necessary for degrading mucin sugars. The final aim of our study, addressed in manuscript IV was to determine if *E. histolytica* secreted glycosidases are involved in mucin degradation. Our overall studies in this thesis did not initially include the investigation of the effect of parasite glycosidase activity on mucin. But during our initial attempt to characterize mucinase activity, we made some crucial observations. We discovered that degradation of ^{3}H-labeled mucin oligosaccharides could not be inhibited by E-64 or any other protease inhibitor used in the studies [11]. This led us to suggest that

mucin oligosaccharide degradation could be contributing to the destruction of the mucus gel. We also theorized that this degradation could expose the mucin core to proteases and contribute to increased degradation of the polymer. The specific enzymes involved in this event still remain to be determined.

In conclusion, the results of this study have demonstrated for the first time that *E. histolytica* cysteine proteinases are involved in the initial events of invasive amebiasis through disrupting the first line of innate host defense, the mucus barrier. The proposed model of amebic invasion is illustrated in **Fig. 1**.

Figure 1. Model of *E. histolytica* overcoming the innate defense of the colonic mucus barrier during invasive amebiasis.



The initial events leading to invasive disease can be summarized as follows; 1) adherence of the parasite to colonic mucin oligosaccharides, 2) colonization of the mucus layer, 3) degradation of mucin and disruption of the mucus gel, 4) adherence to the colonic epithelium and invasion of the mucosa and 5) hyper-secretion of mucus in response to parasite secreted components. These destructive parasite molecules play many roles in amebiasis from immune evasion to tissue destruction and are now implicated in destroying innate defenses of the gastrointestinal tract. The development of inhibitors against these virulence factors may prove useful in combating this disease.

Our study has raised some interesting questions regarding differences between invasive and noninvasive infection with *E. histolytica*. Why do only 10% of individuals infected with the parasite develop invasive disease? This question still remains to be answered. One could speculate that there are differences between the parasites that invade, such as increased expression of virulence factors. Reports of clinical isolates collected from patients with amebic colitis or liver abscess suggest this is possible since these parasites show an increase in cysteine proteinase activity compared to those collected from individuals with noninvasive disease [12]. There may also be differences in other virulence factors as well. In addition, host factors could also play a role in the outcome of the disease. It is possible that differences in MUC2 between individuals could contribute to infection. There are two major alleles of the *MUC2* gene that encode the protein and they have major differences in the length of the VNTR region, resulting in a "long" and a "short' allele. Subsequently, the corresponding proteins encoded by these genes are different but the consequence of this difference on protein function is unknown. Glycosylation patterns on mucin can differ between individuals and may change the composition or properties of the mucus, although there is currently no evidence to support these theories. In the future, it will be necessary to analyze both differences in mucin between infected individuals as well as variations in virulence factors of different isolates of *E. histolytica*. It would be particularly interesting to identify any polymorphisms in the MUC2 flanking regions that are susceptible to cleavage by the cysteine proteinases. Finally, we can conclude that the factors contributing to invasive amebiasis are multifactorial and much research still needs to be conducted to determine

why only a small percent of infections are invasive. This body of work has advanced the study of amebiasis by deciphering the mechanism of how the parasite invades.

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Appendix

The Thesis Submission Guidelines state that: " If the research for the thesis involved human participants, animal subjects, microorganisms, living cells, other biohazards, and/or radioactive materials, the appropriate compliance certificates must be included as an appendix to the thesis".

The required documents are added as an appendix in the following pages.

McGill University Internal Radioisotope Permit

20050225

Permit Holder & Position	Building (Office)	Building (Lab)	Laboratory Classification		
RIS DR. CHADEE, ASSOCIATE PROFESSOR	INSTITUTE OF PARASITOLOGY	INSTITUTE OF PARASITOLOGY	INTERMEDIATE	**PLEASE POST**	Date Issued 2004/09/01
Department: ARASITOLOGY	Room Number(s): B1.14 — Telephone: 398-7721	Room Number(s): B1.1, B1.2 — Telephone: 398-7997			Expiry Date 2005/08/31

PERSON(S) APPROVED TO WORK WITH RADIOISOTOPES

Name	Train.	Cond(s)	Class(es)	Radioisotope(s)
THY KELLER, TECHNICIAN	Y	2, 4	2, 3	C-14, H-3, P-32
SRINIVAS, GRADUATE STUDENT	Y	2, 4	2, 3	H-3, P-32, S-35
RCY MONCADA, GRADUATE STUDENT	Y	2, 4	2, 3	C-14, H-3, P-32
THERINE IVORY, GRAD STUDENT	Y	2, 4	2, 3	H-3, P-32, S-35
Y DAWN STILLAR, GRAD STUDENT	Y	2, 4	2, 3	H-3, P-32, S-35
BALLERO CELIA, GRAD STUDENT	Y	2, 4	2, 3	H-3, P-32, S-35
INIVAS KAMMANDIMINTI, PHD STUDENT	Y	2, 4	2, 3	P-33, H-3, P-32, S-35
RANIL DEY, POST DOC FELLOW	Y	2, 4	2, 3	P-33, H-3, P-32, S-35
JEUNE VIRAPIN MANIGANDAN, GRAD STUDENT	Y	2, 4	2, 3	C-14, H-3, P-32

GENERAL LICENCE CONDITIONS (OPEN AND/OR SEALED SOURCES)

- The permit must be posted with the CNSC safety poster in the permit holder's premises.
- Radioisotope handling shall be in accordance with the McGill Radiation Safety Policy Manual.
- The permit holder must ensure that all persons mandated to work with radioisotopes be properly trained in radiation safety prior to start of work.
- Radioactive work areas must be clearly identified with radiation warning signs.
- Smoking, eating, drinking, storage of foods or drink and the application of cosmetics and contact lenses are prohibited in areas where radioisotopes are used.
- All procedures involving radioactive materials should be carried out on spill trays or on benches lined with disposable absorbent material.
- Procedures that might produce airborne radioactive contamination should be carried out in a functioning fume hood.
- When hand or clothing contamination is possible, protective gloves and clothing must be worn.
- After handling radioactive material and especially before leaving the laboratory, personnel must ensure that all parts of their cloths are not contaminated.
- Purchase and disposal of radioisotopes must be kept electronically or documented in a log book.
- For disposal of radioactive waste, consult the McGill Radiation Safety Policy Manual and/or McGill WMP.
- Wipe tests must be performed and records be kept in a log book.
- The permit must reflect the exact conditions under which radioactive material is used. If changes must be made, contact the RSO at 398-1538.
- The device(s) containing the sealed source(s) must have a radiation symbol and an identification label bearing the name and telephone number of the permit holder.
- Leak tests must be performed on sealed sources equal to or greater than 50 MBq (1.35mCi).
- Extremity dosimeter (i.e. ring or wrist badges) must be worn if 50 MBq or more of P-32, Sr-89, Sr-90 & Y-90 are used.
- Workers using I-125 or I-131 on open bench (5 MBq), in a fume hood (50 MBq) or a vented glove box

Approved Unsealed Radioisotope(s) and Location(s)

Isotope	Possession Limit	Stored		Handled
-3	< 400 MBq (11 mCi)	B1.1		C019,B1.1,A110,C022
-14	< 10 MBq (270 uCi)	A003		C019,B1.1,A110
-32	< 1 GBq (27 mCi)	A003		C019,B1.1,
-35	< 400 MBq (11 mCi)	A003		C019,B1.1,A110,C022
-33	< 400 MBq (11 mCi)	A003	FREEZER	C019,B1.1,C026

Approved Sealed Radioisotope(s) and Location(s)

Permanently Housed Source(s)				*Accessible Source(s)*			
Isotope	Activity	Stored	Handled	Isotope	Activity	Stored	Handled

ersonnel Conditions

.Must attend thyroid bioassays within 5 days of use if 50 MBq (1.35 mCi) of I-125 are anipulated in a fume hood.
.Must wear a whole-body film badge, if gamma, x-ray or high energy beta emitters are used.
.Must wear an extremity TLD dosimeter, if more than 50 MBz (1.35 mCi) of P-32, Sr-89, Sr-90 or -90 are used.
.Classified as Radiation User.
.Classified as Nuclear Energy Worker (NEW).
.Does not work with any radioisotopes but may be indirectly exposed.

Workload Classes

1 .Work load < 10MBq (270 uCi) of unsealed radioisotopes in open areas.
2 .Work load < 10MBq (270 uCi) of unsealed radioisotopes in a fume hood.
3 .Work load > 10MBq (270 uCi) of unsealed radioisotopes in open areas.
4 .Work load > 10 MBq (270 uCi) of unsealed radioisotopes in a fume hood.
5 .Work with sealed sources.
6 .Individual does not work with radioactive sources but normal working conditions involve presence in a room where radioactive material is used or stored.

Joseph Vinetti — Nov 17/04
RSO & Occupational Hygienist — Date
McGill Environmental Health & Safety

For: Dr. Ian Butler, Chairperson
McGill University Laboratory Safety Committee
Associate Vice-Principal (Research)

McGill University

University Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

project should be commenced without prior approval of an application to use biohazardous materials. Submit this lication to the Chair, Biohazards Committee, one month before starting new projects or expiry of a previously roved application.

PRINCIPAL INVESTIGATOR: Kris Chadee TELEPHONE: 398-7721

ADDRESS: Institute of Parasitology, FAX NUMBER: 398-7857

E-MAIL:kris_chadee @maclan.mcgill.ca

DEPARTMENT: Parasitology

PROJECT TITLE: Colonic mucin biosynthesis, secretion and function in infectious diseases

---

FUNDING SOURCE: CIHR xx NSERC NIH FCAR FRSQ

INTERNAL OTHER (specify) _____

Grant No.: 206-14 Beginning date: 01/07/03 End date: 30/06/04

Indicate if this is

X Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.
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Investigator Data:

cipal Investigator:	Kris Chadee	**Office #:**	398-7721
artment:	Parasitology	**Fax#:**	398-7857
ress:	Macdonald Campus	**Email:**	kris_chadee@maclan.mcgill.ca

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ıe:	Dr. Kris Chadee	**Work #:**	398-7721	**Emergency #:**	695-2534
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ir, Facility Animal Care Committee:		**Date:** 16/04/03
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ir, Ethics Subcommittee(as per UACC policy):		**Date:**
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This protocol has been approved with the modifications noted in Section 13.

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CERTIFICATE

This is to certify that Darcy Moncada can use the results obtained during our collaborative project in her thesis.

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Ph.D. student

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From: Dr. S. Ankri
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