PH DEPENDENT PROTEOLYSIS OF NEURAL CADHERIN

by
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DEDICATION

I would like to dedicate my thesis and the hard work it required to my great-grandmother, Margie Nell Hutto Jones, who passed from this world on April 6, 2013.
I would like to thank Dr. Pedigo for her unceasing commitment to my education during my time at Ole Miss. Whether the learning occurred in the classroom or in the lab, Dr. Pedigo pushed me farther than I would have gone on my own. Her dedication has greatly enriched my education and experience as an undergraduate researcher. Furthermore, I would like to thank Nagamani Vunnam, Xiaoyun Howard, Jared Jungles, and Matt Duke for their assistance in my research. A special thanks is awarded to my lab partner for a year and a half, René Phongham. Without her help, stumbling through the many months as a research student would have been a lot more difficult. Additionally, I would like to thank my family for their unconditional support while I was writing my thesis. Above all, I would like to thank the Good Lord for blessing me with the opportunity to further my education at Ole Miss.
ABSTRACT

CANDICE ASHTON WALTERS: pH Dependent Proteolysis of Neural Cadherin
(Under the direction of Dr. Susan Pedigo).

Cadherins are the primary calcium-dependent cell-cell adhesion molecule and are essential to vertebrates. They ensure the integrity of epithelial barriers that allow for maintenance of homeostasis in humans and protection against invading microorganisms. A common invader capable of forging past this line of defense is the fungus Candida albicans. The main virulence mechanism of C. albicans in acidic environments is its secreted aspartyl proteinases (SAPs), which are hydrolytic enzymes that degrade cadherin-mediated cell adhesion leading to infection of the underlying tissues. Cadherin is normally resistant to cleavage by proteases due to stabilization of the protein structure by bound calcium and the high levels of calcium in the extracellular space. The binding of calcium to cadherin requires intact, functional calcium binding sites comprising clusters of carbonyls and negatively charged carboxylates. Thus, there are two main environmental factors that contribute to the ability of the SAPs to degrade cadherins in vivo: low pH protonates acidic carboxylates in the calcium binding sites, thereby decreasing the calcium binding affinity, and the dysregulated calcium concentration in the acidic lumen of the host tissue. This thesis investigates the pH and pCa dependence of protease susceptibility of Neural-cadherin (N-cadherin). The model protease used here is Endoproteinase Glu-C. As predicted, we observed an increase in susceptibility with a decrease in pH and an increase in pCa. There is one stable fragment that is formed after
exposure to the protease with an apparent size of ~10,000 D based on migration in SDS-PAGE. Scanning the sequence of N-cadherin identified possible cleavage sites. Future studies will reveal the identity of this stable fragment.
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LIST OF ABBREVIATIONS

AAPF   N-Succinyl-Ala-Ala-Pro-Phe-p-Nitroanilide
AIDS   Acquired Immune Deficiency Syndrome
APS    Ammonium Persulfate
BTEE   N-Benzoyl-L-Tyrosine Ethyl Ester
cDNA   Complementary DNA
D      Aspartate, Aspartic Acid
DTT    Dithiothreitol
E      Glutamate, Glutamic Acid
EC1, EC2 Extracellular Domain 1, Extracellular Domain 2
ECAD   Epithelial Cadherin
ECD    Extracellular Matrix
F      Phenylalanine
H      Histidine
HOAc   Acetic Acid, CH₃COOH
HEPES  4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid
HIV    Human Immunodeficiency Virus Infection
MES    2-(N-morpholino)ethanesulfonic acid
MMP    Matrix Metalloproteinase
NCAD   Neural Cadherin
NCAD12 Neural Cadherin, domains 1&2
PCR    Polymerase Chain Reaction
RHE    Reconstituted Human Epithelium
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<tr>
<td>TEMED</td>
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<td>Tris</td>
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</tr>
<tr>
<td>W</td>
<td>Tryptophan</td>
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<td>Y</td>
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INTRODUCTION

Cadherins are essential molecules to vertebrates. As the primary transmembrane protein present in adherens junctions, they are in essentially every cell in animals from nematodes to humans (1). Higher order organisms generally contain various types of cadherins. In particular, epithelial cadherin (ECAD) plays an important part in epithelial cell adhesion contacts. ECAD also plays a key role in signaling pathways that regulate cell proliferation, differentiation, and survival (2). Additionally, neural cadherin (NCAD) is involved in numerous aspects of neural development including synaptogenesis, synapse maintenance (3, 4), and regulation of synaptic plasticity (5, 6). Furthermore, both ECAD and NCAD are important in synapses of neurons, where they become important in the formation of memories with long term potentiation (7).

The structure of these integral proteins is critical for their function. In general, they have five homologous extracellular domains, one segment spanning the membrane, and a C-terminal region in the cytoplasm that is anchored through catenins to cytoskeletal actin microfilaments, thus forming adherens junctions (1) (**Figure 1**). Therefore, the cytoplasmic region is critical for communication with the actin cytoskeleton (8). EC1, the most distal extracellular domain, dictates binding specificity because it is the domain through which cadherin makes contact with other cells. Interactions between the participant protomers are called dimerization and adhesion; both are calcium dependent.
Without calcium, cadherins are incapable of forming adhesion complexes and the molecule is monomeric (1, 9).

**Figure 1. Illustrations of Classical Cadherin Domain Structure.** (A) Schematic drawing of classical cadherin. Cadherin is a single polypeptide with ~ 650 amino acids. Modular EC-domains are represented as blue ovals with three calcium ions (orange) bound at the interface between each modular domain. The cytoplasmic domain is shown with cytoplasmic proteins called catenins that interact with the actin cytoskeleton. (B) Overall structure of classical cadherins. The extracellular domains (EC1 - EC5) are shown in ribbon diagram (orange) with calcium ions in green (pdb-ID: 1L3W,(10)). The transmembrane segment is shown as solid orange line passing through the bilayer membrane. The structure of some segments of the intracellular domain is unknown (orange dotted lines). The structure of the cytoplasmic proteins p120- (green) and β-catenin (blue) have been determined. β-catenin interacts with actin filaments through α-catenin.

Calcium binds at the interface between each of the five EC-domains. A short linker segment between each domain contains amino acids that form the calcium binding sites. The amino acid sequences for E- and N-cadherin are shown in **Figure 2.** The conserved calcium binding residues are marked. These calcium binding regions are highly conserved structurally between each successive domain interface and between the various forms of cadherins. The binding of three calcium ions in the linker region
between EC1 and EC2 induces a conformational change in the molecule from a mobile state to a relatively fixed state. The calcium ions allow carboxylate side chains to be brought together to form stabilizing intermolecular bonds with the calcium ion itself. Removal of calcium results in the elimination of these interactions, leaving the domains of each cadherin molecule connected by flexible linkers (1).

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**Figure 2.** Amino Acid Sequence of E-cadherin and N-cadherin. The amino acid sequences of E- and N-cadherin from mouse are aligned to illustrate similar (.) and identical (:) amino acids in the first two EC-domains of the proteins using LALIGN (11). Residues involved in binding calcium at the interface between EC1 and EC2 are marked with the symbol § and colored blue (EC1), black (Linker 1) or red (EC2). Residues highlighted in yellow are the linker regions. Histidines in N-cadherin are highlighted in red and tryptophans in green.

We expect that the pH of the extracellular environment will affect the stability of adherens junctions. A decrease in pH will cause a change in the protonation state of amino acid side chains that compose the cadherin protein. The change in protonation is the result of $H^+$ ions being added to or removed from the side chains of the residues composing the protein. If the pH is less than the pK$_a$ value for an amino acid, then the protonated form of the amino acid’s side chain predominates according to the Henderson-Hasselbalch equation. If the pH is greater than the pK$_a$ value, then the deprotonated form of the amino acid side chain predominates. The pK$_a$ of the imidazole group composing
histidine’s side chain is approximately 6 and the pKₘ of the carboxylate side chain on glutamate is approximately 4.25 (12). The actual value of pKₘs for residues in proteins can vary significantly from these canonical values (13). Therefore, a change in the pH in the environment of a protein has the potential to create drastic differences in the protonation state of residues within a protein. For this precise reason, a protein’s delicate structure is highly susceptible to changes in pH (14, 15).

Furthermore, a decrease in pH can significantly affect the stability of a protein by changing the magnitude of ionic interactions. Ionic interactions are essential in order to establish a protein’s correct tertiary and quaternary structure. Without the attractions between oppositely charged residues or the repulsion that results from like charges on residues, a protein will not be able to fold correctly and thus will lose its function. Acidic proteins, like cadherins, will experience electrostatic repulsion at neutral pH due to the negative charges on the acidic side chains. The negative charges at neutral pH are the result of deprotonation of side chains because the pH has exceeded the acidic residue’s pKₘ. Thus, the structure of an acidic protein may be stabilized by a decrease in pH due to a decrease in electrostatic repulsion from neighboring acidic residues (16, 17). The structure of proteins may also decrease in stability as the pH is decreased presumably due to a decrease in electrostatic attraction between oppositely charged amino acid side chains like lysine and glutamate (16). Attraction and repulsion are both major factors contributing to the overall structure of a protein. Changes in pH that result in an imbalance of charges thus change the structure and function of a protein. These types of changes have the potential to wreak havoc with essential proteins such as cadherin
because a change in its structure results in an increase in an organism’s susceptibility to invasion by pathogenic organisms.

In general, cadherin is an acidic protein. For experimental purposes, only the two extracellular domains of neural cadherin are used (EC1 and EC2) and these two domains alone have 15 negative charges at pH 7.4. The result of so many negative amino acid residues is significant electrostatic repulsion in the Apo-state, particularly in the calcium binding pocket at the interface between adjacent EC domains. Calcium binding stabilizes the domains in part by reducing electrostatic repulsion. Therefore, the high affinity that cadherin demonstrates for calcium ions in these pockets is largely the result of the attraction between the negatively charged side chains and the positively charged calcium ion. This concept was illustrated in studies by Prasad et al (18), where the addition of NaCl significantly stabilized the Apo state of EC2 constructs of E-cadherin. Similar studies were performed by Vunnam et al on EC2 constructs of N-cadherin, indicating that destabilization by electrostatic repulsion is a general phenomenon in classical cadherins (19).

In order to orient the reader to aspects of the N-cadherin sequence, discussion of what is known about the various sequence features in N-cadherin is needed. There are three histidines (H), two in EC1 (H75 and H79) and one in EC2 (H110). H110 is only two residues away from a tryptophan (W) residue, W113. Protonation of histidine residues is known to decrease the fluorescence of neighboring tryptophans (20). There are nine glutamates at positions 11, 20, 69, 89, 107, 119, 167, 181 and 196. E11 and E69 offer one of their side chain oxygens to calcium in site 1 and the other to the calcium in site 2 (21). The side chain of E89 is important in forming a salt bridge with the N-
terminus in the closed monomer and closed dimer structures (22, 23). Mutation of W113 to alanine decreases the calcium binding affinity of N-cadherin (22). Mutation of W2 to alanine decreases the stability of EC1 and completely abrogates dimerization (22, 24).

The importance of this molecule is evident when examining its adhesion capability. The structure of each cadherin monomer allows for it to make adhesion contacts with one cadherin on another cell (EC1-EC1) and lateral contacts with two cadherins on the neighboring cell (EC1-EC2) (10) (Figure 3). This side-by-side association between the first domains of the different cadherin molecules can be repeated to form a double-weave structure. This double-weave structure is important because cells become interlocked via EC1-EC1 interactions. Individual interactions are relatively weak, but due to polyvalency, there is tight binding between adjacent cells (10). Therefore, the maintenance of these cell adhesions is highly important in order to support the integrity of the epithelial and endothelial barrier and therefore prevent the entry of foreign invaders.
Figure 3. Diagram Representing Cadherin’s Double-Weave Structure. Cadherin proteins are capable of forming two types of adhesions. Cadherin forms two EC1-EC2 lateral contacts with adjacent cadherin molecules and one EC1-EC1 contact with a cadherin on another cell (Boggon, 2002). Through repetition of both types of adhesions, many cadherin molecules ultimately give rise to a double-weave structure that provides protection against invasion. Adapted from Boggon et al (10).

A common invader is Candida albicans, which is the most common fungal pathogen in humans. Typically, Candida albicans is a normal commensal organism living in the vagina, gastrointestinal tract, and oral cavity of humans (25, 26). However, under certain circumstances, such as when the levels of microflora become imbalanced or when host defense mechanisms are impaired, C. albicans can proliferate and become capable of causing serious host damage through vaginal, cutaneous, oral, or systemic candidiasis (27). Candidiasis is an incredibly common problem in HIV patients because their immune system is impaired. Also, nearly three-quarters of healthy women suffer from at least one vaginal yeast infection while about 5% suffer from recurrent infection (25). However, systemic candidiasis is the most serious type of infection due to its high
mortality rate ranging from 33% to 54% and high incidence of sickness and disease in those who do survive (26, 28). The incidence of Candida infections has continued to steadily increase over the past decades and this trend is predicted to continue due to increasing drug resistance to the few effective antifungal drugs available (29). Actually, candidiasis has become the fourth most common cause of noscomial bloodstream infections in America. Despite its common occurrence, there are very limited numbers of suitable and effective antifungal drugs to combat these infections. Therefore, research into Candida albicans’ pathogenic mechanism is crucial (25).

In order for Candida to make the transition from commensal yeast to pathogenic agent, it must first adhere to host cells and enter the blood stream (26). Numerous studies have highlighted several mechanisms that would allow for Candida to exit circulation and invade tissues, however the specifics of the mechanisms remain to be elucidated (25, 26, 28, 30). The most well studied pathways of invasion include transmigration, incision, gapping, and proteolysis (Figure 4). In transmigration, the pathogen of interest possesses specific proteins that bind to the host cell’s adhesion molecules, which promotes its own endocytosis (31). The endocytosis of the pathogenic agent is accomplished either through a clathrin-mediated or caveolin-mediated pathway (32-34). The virulent pathway of incision is accomplished through rapid growth of the hyphae form of the Candida fungus. Its accelerated growth effectively pierces the host epithelial layer, which leads to the death of the host cell and entry of the organism into the tissues of the host (35, 36). Gapping is a mechanism by which the pathogen’s proteins bind to cell-cell and cell-matrix adhesion proteins moving in a hand-over-hand mechanism to allow entry between adjacent host cells in a similar fashion to the interaction of immune cells of the
Finally, the most widely studied mechanism includes the secretion of proteinases that allow for the degradation of adhesion molecules. The destruction of these molecules thus increases the porosity of the epithelial or endothelial barrier that allows for easier pathogen entry into the blood stream (26).

![Diagram](image.png)

**Figure 4.** Schematic of Different Pathogenic Pathways used by *Candida albicans*. A figure illustrating the various possible methods of invasion through human tissues utilized by the fungus *Candida albicans*. Each method owes its pathogenecity to a variety of virulence factors. This paper focuses on the proteolytic mechanism through the use of SAPs. However, the action of SAP proteins could contribute to each stage of infection. Adapted from (26).

The focus of this research lies within the realm of the virulent pathway of infection through secretion of proteolytic enzymes. *Candida* species, like most pathogenic microorganisms, have virulence factors as a mechanism to successfully infect a host (25, 28). One of *Candida albicans* most significant virulence factors is a variety of extracellular hydrolytic enzymes it is capable of producing. The most studied and well known of these enzymes is *Candida albicans’* secreted aspartic proteinases (SAPs) (37). The family is named for the Aspartic Acid residue in its active site. Aspartyl proteinases are very diverse and have a broad substrate specificity, which allows for them to break
down a wide range of proteins in the human body (25). Studies have made it possible to determine that SAPs are capable of degrading proteins integral for immune defense and cell integrity such as “mucin, extracellular matrix proteins, numerous immune system molecules, endothelial cell proteins, and coagulation and clotting factors” (25). *C. albicans’* aspartyl proteinases comprise a family of isoenzymes that are encoded by at least 10 different SAP genes (38). Using *in vitro* experiments, these genes have been shown to be regulated differentially and to possess unique roles that are important at different times during the infection process and in different types of infection. The pH dependence of the proteolytic activity of SAPs varies with the optimum pH between pH 2.0 and 7.5 depending on the isozyme (39-41). While the substrate specificity of SAP isozymes is not known, they are structurally similar to Pepsin (42), which has a preference for bulky hydrophobic amino acids (43). Recent studies have reported that SAP5 degrades E-cadherin at pH 4 (30, 44). Our argument is that not only is SAP5 more active at low pH, but also that cadherin is more vulnerable to proteolysis due to decreased calcium binding affinity.

Studies by Schaller et al suggest that the proteinase activity of SAPs strongly contributes to colonization, infection, and tissue damage in the in vitro model (37). These studies were conducted using reconstituted human epithelium (RHE) and animal models such as mice. The importance of SAPs in establishing the various forms of candidiasis was demonstrated by using an inhibitor of the aspartic proteinases, pepstatin A. Animal models were used to establish the importance of SAPs in systemic and vaginal candidiasis; RHE was used to elucidate SAPs’ importance in establishing oral infections. The results of the pepstatin experiments showed a dramatic decrease in the
extent of lesions caused by a strain of *C. albicans*; thus indicating that aspartic proteinase inhibitors demonstrate a protective effect (37). These studies together are consistent with the conclusion that the proteinase activity of SAPs contributes to colonization, infection, and tissue damage (37). Therefore, these studies promise a significant importance in establishing an accurate relationship between the virulence mechanism of *Candida albicans’* secreted aspartic proteinases and the occurrence of candidiasis in human tissue.

Just as the integrity of the epithelial barrier protects the host from pathogens, it is also required to prevent metastasis of cancer. Almost all members of the cadherin superfamily are affected by cancer through the disruption of the organized adhesion of tissues. The disruption of the cellular adhesion is accomplished through multiple means including loss of contact inhibition, altered stromal interactions and cell migration, and through genetic and epigenetic changes that thus result in changes in cell signaling (45). Furthermore, proteolysis of cadherin is of great importance in the oncogenesis of cancer (45).

Matrix metalloproteinases (MMPs) are the enzymes responsible for the cleavage of many proteins, including cadherins, which ultimately result in tumorigenesis. MMP is typically overexpressed in cancerous human tissues and is associated with the progression of cancer. Tumorigenesis is a multi-step process that includes cell growth, cell invasion, metastasis of cells, and angiogenesis (46). MMPs have roles at every step of tumorigenesis, whether they are involved in altering proteins associated with the extracellular matrix (ECM) or non-ECM proteins (46). Degradation of ECM components by MMPs not only includes processes involved with tumor invasion and metastasis, but it also has expanded roles where they are necessary for the creation and maintenance of an
environment that allows and facilitates angiogenesis and growth of both primary tumors and tumors at metastatic sites (46). Additionally, certain MMPs are involved in regulating activities of molecules that are associated with the physiology of the cell and shedding of the cadherin ectodomains (Figure 5). Ectodomain shedding, which is pertinent to cadherin molecules, is a process that results in the proteolytic removal of the extracellular domain of a transmembrane protein (46). The two main MMPs associated with the cleavage of cadherins are MMP-3 and MMP-7. MMP-7, also known as matrilysin, is involved in multiple other oncogenetic processes (46). Because of the disruption of cellular adhesion that would result from the cleavage of the extracellular domain of cadherins, ectodomain shedding is highly tied to tumor invasion. Overall, there is a correlation between the expression level of MMP and the stage of the tumor in its progression.

**Figure 5.** Detrimental Effects of the MMP Matrilysin on Cadherin Cellular Adhesions. The figure illustrates the effect of MMPs, specifically matrilysin, on the cellular adhesions between cadherin molecules (46). The simplified representation of the molecular double-weave structure formed between cadherin molecules is cleaved by matrilysin, thus resulting in the disruption of the cadherin complex formed between cells.
The detrimental effects of cancer caused by proteolysis through MMPs are enhanced through the disruption of intracellular and extracellular pH (47). Dysregulated pH is an emerging hallmark of cancer because in cancer cells the intracellular pH (pHi) is higher than normal whereas the extracellular pH (pHe) is lower than normal. In normal adult cells, the pHi is 7.2 and is typically lower than the extracellular pH, which is normally ~7.4. However, in cancerous cells, the pHi is much higher than normal with a pH ≥ 7.4, while the pHe ranges from ~6.7 to 7.1 (47).

This reversed pH gradient, which is becoming highly recognized for its adaptive features for cancer, is responsible for promoting oncogenesis through means such as evading apoptosis, metabolic adaptations, promoting cellular proliferation, migration and invasion (47) (Figure 6). Specifically, the lower pHe has a direct effect on the remodeling of the extracellular matrix and stimulating acid-activated proteases to promote tumor invasion and metastasis, which were previously discussed in relation to MMPs. Therefore, the lower extracellular pH in combination with the higher intracellular pH creates a ‘perfect storm for metastatic progress” (47).
Figure 6. Cancer Induced Effects of pH. In comparison to the normal pH of the human body, the reversed pH seen in cancerous tissues causes a multitude of negative consequences. A higher intracellular pH (pH$_i$) contributes to tumor dysplasia and ultimately to cell migration through changes within the cell. A lower extracellular pH (pH$_e$) promotes metastasis and invasion of tumorigenic cells to secondary sites within the body (47).

Furthermore, the lower pH decreases cadherin’s binding affinity for calcium. A lowered affinity for the ion essential for cadherin’s molecular double weave structure would thus facilitate the ability of tumors to invade and metastasize to other tissues and the disassembly of synaptic junctions of neurons. Therefore, it can be concluded that a lower pH has the potential to create drastic changes within human cells and in their environment that lead to extreme changes in their physiology and behavior.
Specific Aims

1. Develop a proteolysis experiment to assess the effect of pCa and pH on stability of cadherin.

2. Test the hypothesis that the calcium binding affinity should decrease with a decrease in pH.

Significance

Alteration in pH is known to happen in several significant human pathological conditions including cancer and opportunistic infections in immunocompromised patients (47, 48, 49). Lowering of pH can affect a number of aspects of cell biology. Here, we are concerned over the effect of decreased pH on cell adhesion. In cancer, it leads to the circulation of metastatic cells from the primary tumor, and their penetration of the epithelial barrier to form secondary tumor sites (47). In immunocompromised patients, a lower pH causes porous adhesion among cells, apparently by lowering cadherin’s affinity for calcium and thus decreasing the strength of adherens junctions and allowing for easier pathogen entry (26). Studies here are directed toward developing a proteolytic method for assessing the stability of adherens junctions.

Approach

Neural cadherin’s time-dependent, calcium-dependent, and pH-dependent susceptibility to proteolysis by α-Chymotrypsin, Pepsin, and Endoproteinase Glu-C were observed through qualitative analysis of protein cleavage via SDS-PAGE.

Proteinases: SAPs, Candida’s primary hydrolytic enzyme, have a general specificity for nonpolar, hydrophobic amino acids. The protease α-Chymotrypsin cleaves proteins on the C-terminal side of tryptophan, tyrosine, and phenylalanine residues. α-
Chymotrypsin’s optimum pH is 7.8. At pH 6.0, α-Chymotrypsin only has 35% of maximal proteolytic activity. Due to its similar specificity, α-Chymotrypsin was utilized in order to simulate proteolysis by SAPs. Pepsin is a member of the SAP family and has a broad specificity but demonstrates the same preferential cleavage for nonpolar, aromatic amino acid residues. However, it has a much lower optimum pH range of 2.0-4.0. Endoproteinase Glu-C has specificity for cleaving peptide bonds on the C-terminal side of the acidic residue glutamate and aspartate. It displays activity over a broad pH range of 3.5 to 9.5 and exhibits maximal activity from pH 4.0 to 7.8. This variety of proteases allows a comprehensive look into several aspects of Candida’s SAPs.

Analytical Technique: SDS-PAGE was used to assess NCAD’s susceptibility to proteolysis by α-Chymotrypsin at several times points in addition to its susceptibility to proteolysis by Pepsin and Endoproteinase Glu-C at varying Ca$^{2+}$ and H$^{+}$ concentrations at several time points. SDS-PAGE was carried out using 15% Tris-Tricine gels. These gels allow for optimization of the resolution of the low molecular weight peptide fragments. Additionally, 17% Tris-Glycine gels were used to visualize the enzymatic assays.

Buffers: The effect of pH on proteolytic activity was assessed using several biological buffers with a pH between 6.0 and 7.4, which are similar to the physiological situations mentioned previously.

Cadherin: The first two modular domains of neural cadherin (NCAD12) were used in the proteolysis studies. NCAD12 contains an intact Ca$^{2+}$ binding pocket and shows Ca$^{2+}$-mediated dimerization in previous studies (8). The two extracellular domains are
regarded as the minimal functional adhesion unit. Therefore, if cell adhesion is disrupted by protease, it is the NCAD12 portion that would be cleaved.
MATERIALS

Plasmid Construction

The cDNA of mouse N-cadherin was provided by Dr. L. Shapiro (Columbia University, USA) and was used as a template for PCR amplification of the gene for the first two domains (residues 1-221), designated as NCAD12. PCR amplification of the two-domain fragments, digestion of template DNA with restriction enzymes, ligation of the fragments into the pET30 Xa/LIC expression vector (Novagen), and subsequent transformation into *E. coli* (DE3) expression cells were performed according to standard protocols, utilizing KOD HiFi DNA Polymerase (Stratagene) and Xa/LIC cloning kit (Novagen). The full-length genes were sequenced to confirm absence of mutations (50).

Expression and Purification

Protein expression and fractionation of inclusion bodies are described previously. The resulting pellets were suspended in denaturing His Tag binding buffer (6 M urea, 20 mM Tris/HCl, 0.5 M NaCl, 5 mM Imidazole, pH 7.5). Supernatants were applied to a Ni affinity column (GE Life-Sciences). The protein was eluted with 10 mM Tris/HCl, 250 mM NaCl, 0.5 M Imidazole, pH 7.9. The elution fractions containing NCAD12 were dialyzed in 140 mM NaCl, 20 mM Tris, 5 mM CaCl$_2$, 1 mM DTT, and 5% glycerol, 7.4. Immobilized trypsin (Pierce) was used to remove the 45 residue N-terminal affinity label. Digested proteins were dialyzed against 140 mM NaCl and 10 mM HEPES, pH 7.4 (SEC Buffer). Protein stocks were aliquoted and stored at 4°C. All protein stocks were stored
in the apo state. The extinction coefficients were determined experimentally and found to be $17700 \pm 500 \text{ M}^{-1}\text{cm}^{-1}$ for monomeric NCAD12 (50). Purity of proteins was assessed by SDS-PAGE as discussed below. To obtain proteins of pH 6.0 and 7.0, one mL pH 7.4 stock was dialyzed in buffers of the respective pH. The composition of buffers is shown in Table 1.

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<td>140 mM</td>
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</tbody>
</table>

### Proteinases

One of the proteinases used for this experiment was $\alpha$-Chymotrypsin, type II, from bovine pancreas, which is essentially a salt-free, lyophilized powder. The activity is 51 units/mg protein. It was stored at a temperature less than 0°C. Preparation of the 0.04 mg/mL Chymotrypsin stock was achieved by dissolving 0.7 mg of $\alpha$-Chymotrypsin to 17.5 mL of SEC buffer, resulting in the desired enzyme concentration. Its pH optimum is 7.8, but shows activity in a wide range of pH. $\alpha$-Chymotrypsin is a serine protease that preferentially cleaves on the C-terminal side of the aromatic amino acids tyrosine (Y), phenylalanine (F), and tryptophan (W).

Another proteinase utilized was Pepsin, which is from porcine gastric mucosa, which is essentially a salt-free, lyophilized powder. It was stored at a temperature less than −20°C. Preparation of the 0.01 mg/mL stock at pH 3.67 was carried out by adding 10.0 µL 1.0 mg/mL Pepsin to 990 µL 0.1 M acetic acid (HOAc) stock at pH 3.67. Because Pepsin is a member of the SAP family and due to its optimum pH ranging from 2.0-4.0, it was utilized to observe the proteolysis of NCAD at very low pH. Pepsin shows
relatively nonspecific cleavage but exhibits a preference for cleaving bulky aromatic amino acids, Leucine but not other hydrophobic amino acids (43).

A third proteinase used was Endoproteinase Glu-C, which is from *Staphylococcus aureus* strain V8. It was stored at a temperature less than −20°C. The protein used was previously placed in solution and aliquotted by a previous researcher on October 22, 2004. Preparation of the 0.19 mg/mL stock was accomplished by dissolving dry powder in distilled water. The recommended incubation time for Endoproteinase Glu-C is between 2-18 hours, depending on the enzyme to substrate ratio. It is active over a pH range of 3.5 to 9.5, but exhibits maximal activity between 4.0 to 7.8. Its optimum activity over such a broad range, especially in the range we are investigating, is of particular importance in this study. It is a serine endoprotease that cleaves peptide bonds on the C-terminal side of glutamate (E) and aspartate (D) residues, however cleavage of aspartate is exceptionally rare in acidic proteins. Details of these proteases are summarized in Table 2.

### Table 2. Protease Details

<table>
<thead>
<tr>
<th>Proteinase</th>
<th>Concentration Stock</th>
<th>Buffer</th>
<th>Catalog #</th>
<th>Lot #</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Chymotrypsin</td>
<td>0.04 mg/mL</td>
<td>pH 7.4 Buffer</td>
<td>C-4129</td>
<td>119H7660</td>
</tr>
<tr>
<td>Pepsin</td>
<td>0.01 mg/mL</td>
<td>0.1 M HoAc, pH 3.7</td>
<td>P-6887</td>
<td>73F-8020</td>
</tr>
<tr>
<td>Endo-GluC</td>
<td>0.19 mg/mL</td>
<td>Water</td>
<td>P-2922</td>
<td>072K5100</td>
</tr>
</tbody>
</table>

**Tris-Tricine Gels**

SDS-PAGE was carried out using 15% Tris-Tricine gels (51). These gels allow for optimization of the resolution of the low molecular weight peptide fragments. The gel buffer pH is the same for the separating and stacking gels. 15% Tris-Tricine gels were chosen in order to optimize the resolution of the full-length construct and the low
molecular weight peptide fragments. The gels were made using 15% Acryl/Bisarcrylamide (40%; 19:1) stock solution, 25% of 2 M Tris solution (pH 8.45, 0.4% SDS), 0.5% of APS (10%), and 0.05% of TEMED. Tris-Tricine 2X loading buffer, composed of 1 mL 0.5 M Tris-HCl pH 6.8, 1.5 mL 75% glycerol, 0.4 g SDS, and 1 mg coomassie blue, was used. Gels were run with an anode buffer of Tris Buffer (pH 8.9) and a cathode buffer of Tris-Tricine, SDS solution (pH 8.25).

**Tris-Glycine Gels**

Since the Tris-Tricine gels were underwhelming, we used a standard Tris-Glycine recipe that has acidic pH in the stacking gel and basic pH in the resolving gel. The gels were made using 17% Acryl/Bisarcrylamide (30%; 29:1) stock solution, 25% of 3 M Tris solution (pH 8.8), 0.1% SDS, 5% Glycerol, 0.03% of APS (10%), and 0.05% of TEMED. Stacking gel was 4% Acryl/Bisarcrylamide at pH 6.5. Gels were run with a buffer of Tris (25 mM) and Glycine (190 mM) at pH 8.6.
METHODS

NCAD12’s calcium-dependent, time-dependent, and pH-dependent susceptibility to proteolysis by α-Chymotrypsin, Pepsin, and Endoproteinase Glu-C were observed through qualitative analysis of protein cleavage via SDS-PAGE on 15% Tris-Tricine gels and 17% Tris-Glycine gels. Samples were made with Apo conditions (no Ca\(^{2+}\)), low calcium conditions (10 µM Ca\(^{2+}\)), high calcium conditions (100 µM Ca\(^{2+}\)), and very high calcium conditions (1,000 µM Ca\(^{2+}\)).

Concerning the time-dependent proteolysis by α-Chymotrypsin, digests were made by exposing 60 µL of 45 µM NCAD12 in the Apo state to 15 µL 0.04 mg/mL α-Chymotrypsin. These samples were brought to 82.5 µL with the addition of pH 7.4 Buffer (Table 1). The samples were digested at room temperature (24°C) for 0, 10, 20, 30, 40, 50 and 60 minutes. To quench the reaction, a volume of 10 µL of 2X-SDS-reducing loading buffer was added to 10 µL of sample at each time point and samples were then placed in a boiling water bath for 3 minutes. Each proteolyzed sample, a control sample containing no protease, and an ultra low molecular weight marker were screened via SDS-PAGE on a 15% Tris-Tricine gel at 100 Volts for 70 minutes. The gel was then stained with Coomassie Blue in order to make the peptide bands visible. Qualitative data was discerned through comparison of each time-dependent sample and successive cleavage of bands to the uncleaved NCAD12 in the control. QuantityOne (BioRad) software was used to photograph and view the gel.
The activity of the protease Pepsin was then tested at varying calcium concentrations. NCAD12 stocks at neutral pH were dialyzed against pH 6.0 Buffer. The pH of the protein was decreased even further by diluting 130 µL of 92 µM NCAD12 at pH 6.0 to 130 µL of a buffer of pH 3.7. The pH 3.7 buffer was composed of 6 mL of SEC buffer at pH 7.0 and 2.5 mL 0.1 M HOAc stock. Therefore, the cadherin protein was suspended in a low pH buffer that allowed for the optimum activity of Pepsin to be reached. The varying stock calcium concentrations tested were high calcium at 1,000 µM, medium calcium at 100 µM, and low calcium 10 µM. Digests were made by combining the protein, the low pH Buffer, the various stock calcium concentration, and Pepsin. Table 3 describes the exact volume of each component added.

<table>
<thead>
<tr>
<th>Table 3. Dilutions for the Varying Calcium Digests with Pepsin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Protein</strong></td>
</tr>
<tr>
<td><strong>Buffer</strong></td>
</tr>
<tr>
<td><strong>Ca^{2+}</strong></td>
</tr>
<tr>
<td><strong>Shake</strong></td>
</tr>
<tr>
<td><strong>Enzyme</strong></td>
</tr>
</tbody>
</table>

At the time points of 0 and 30 minutes, 30 µL of each sample were added to 30 µL of 2X SDS-PAGE reducing loading buffer and then boiled in a hot water bath for three minutes. All four proteolyzed samples at each time point along with an ultra low molecular weight marker were screened using SDS-PAGE on 17% Tris-Glycine gel at 200 Volts for 45 minutes. The gel was then stained with Coomassie Blue. QuantityOne Software was utilized to view and photograph the gel. Calcium’s effect on proteolysis was deduced by observation of cleavage-induced peptide bands at each calcium concentration.
Endoproteinase Glu-C’s activity was then tested through varying pH and calcium concentrations. The effect of pH on proteolysis by Endoproteinase Glu-C was observed through the use of several biological buffers that buffer at a range from a pH of 6.0 to 7.4 (Table 1). This range was selected due to its importance in the diseases discussed, such as cancer and candidiasis. HEPES at pH 7.4 and 7.0 and MOPS at pH 6.0 were selected as buffers. Each buffer was made at 40 mM with 140 mM NaCl. Additionally, this experiment tested the effects of calcium concentration at each respective pH. At each pH, protein stocks were prepared that had a final NCAD12 concentration of 45 µM at each of the three calcium levels (Apo, 10 µM, and 100 µM). Therefore, each calcium concentration trial was repeated three times, once at each pH, to test for the combination of these two factors that highly influence the integrity of cadherin. Table 4 lists the various dilutions utilized and Figure 7 provides a visualization of the experiment.

| Table 4. Dilutions for the Varying [Ca$^{2+}$] and pH for Endoproteinase Glu-C |
|---------------------------------|-----------------|-----------------|-----------------|
|                                 | APO             | 100 µM Ca$^{2+}$ | 10 µM Ca$^{2+}$ |
| 45 µM Protein                  | 90 µL           | 90 µL           | 90 µL           |
| Buffer                          | 12 µL           | 12 µL           | 12 µL           |
| Enzyme                          | 21 µL           | 21 µL           | 21 µL           |

**Figure 7.** Diagram of the Varying [Ca$^{2+}$] and pH for Endoproteinase Glu-C. Each calcium concentration was repeated a total of three times, once at each pH of 6.0, pH 7.0, and 7.4. Varying pH in addition to varying the calcium concentration allowed for this experiment to evaluate the combination of the effects of these influential factors on the proteolysis of NCAD12.
At 0 minutes and after an incubation time of 18 hours, 50 µL of each sample was added to 50 µL of 2X SDS-PAGE reducing loading buffer and boiled in a hot water bath for 3 minutes. The various calcium concentrations at each pH were run in a 17% Tris-Glycine gel at 200 Volts for 45 minutes. The gel was then stained with Coomassie Blue and QuantityOne Software was utilized to view and photograph the gel. Calcium’s effect on proteolysis was deduced by observation of cleavage-induced peptide bands at each calcium concentration.
RESULTS

Cleavage with α-Chymotrypsin Increases with Time

The enzymatic assay of α-Chymotrypsin pictured in Figure 8 is indicative that as exposure time increases, cleavage of NCAD12 by α-Chymotrypsin increases. The full-length parent protein is notably a single band in lane 8 at 0 minutes, but, by lane 8 at 60 minutes, there is a primary cleavage band that is only slightly smaller than the parent. This slightly smaller second band indicates that a short peptide was removed by the protease. Additionally, the peptide bands midway through the gel change in abundance as exposure time was increased. Initially, there is a single light band at 0 minutes (lane 2), but it becomes enriched and more stable over the course of the digestion at 60 minutes (lane 8). Notice the doublet of bands that are present even in the uncleaved material of the control. This doublet consists of contaminating peptides formed during the purification procedure, presumably when the fusion protein is exposed to Trypsin to remove the N-terminal fusion.
Figure 8. Cleavage of NCAD12 by α-Chymotrypsin as a Function of Time. Lane 1 ultra low molecular weight standard (Sigma Aldrich), lane 2 exposure for 0 min., lane 3 exposure for 10 min., lane 4 exposure time for 20 min., lane 5 exposure time for 30 min., lane 6 exposure time for 40 min., lane 7 exposure time for 50 min., lane 8 exposure time for 60 min., lane 9 control. As NCAD12’s exposure time to α-Chymotrypsin increases, the intensity of the full-length parent band decreases while simultaneously the lowest fragment band’s intensity increases. This is evident of increased cleavage of NCAD12 by α-Chymotrypsin as exposure time increases. The middle band, the larger fragment peptide, is even present in the control lane (lane 1), therefore, it can be assumed that this is the contaminant in the NCAD12 stock as discussed previously.

Migration of Bands Indicates MW of Protein Fragment

SDS-PAGE gels are useful for determining the size of proteins in a mixture. In this technique, proteins are denatured and made uniformly anionic by SDS and migrate toward the cathode. The extent to which they travel down the gel is determined by the size of the protein and the percentage of acrylamide in the gel. The procedure for using standard proteins to calibrate the relationship between migration on the gel and molecular weight is shown in Figure 9.

The first step to determine the size of a fragment produced during proteolysis is by calculating a R_f value for each of the bands present in the ultra low molecular weight standard. R_f values are calculated by dividing the distance each band travelled down the
gel by the distance measured from the top to the bottom of the gel. This process is shown in Figure 9A. Next, these measured distances are plotted against the log of its molecular weight, which is known (Figure 9B). From this graph, a best-fit line is generated and the equation is used to determine the molecular weight of the fragments produced during proteolysis. Table 5 shows the results of this procedure and lists the calculated molecular weights of the fragments. Notice that the stable fragment’s predicted molecular weight is around 10,000 g/mol. Together, this method allows us to speculate on the identity of the stable fragment shown in the peptide map in Figure 9C. Knowledge of the possible cleavage sites in conjunction with previous studies showing that EC2 is highly susceptible to cleavage, allows us to hypothesize that the stable fragment being formed is from EC1 (18).
Figure 9. Calibration of Gels Using Molecular Weight Standards. Ultra Low Molecular Weight Standards (Sigma Aldrich) were used to calibrate the gels for estimation of molecular weights of protein fragments. (A) R_f values were calculated for each standard band. (B) Experimental R_f values for standard proteins were plotted versus the log of their molecular weight. The linear portion of the curve was fit to a linear equation to resolve values for slope and intercept. (C) Peptide Map for cleavage by α-Chymotrypsin. The top three fragments are primary cleavage fragments. The bottom three fragments are secondary or greater cleavage fragments.

Table 5. Fragments Formed From α-Chymotrypsin Digestion of N-cadherin

<table>
<thead>
<tr>
<th>Band</th>
<th>Rf</th>
<th>Log MW</th>
<th>MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>0.12</td>
<td>4.42</td>
<td>26,000</td>
</tr>
<tr>
<td>1° fragment</td>
<td>0.14</td>
<td>4.37</td>
<td>23,400</td>
</tr>
<tr>
<td>2° fragment</td>
<td>0.22</td>
<td>4.2</td>
<td>15,000</td>
</tr>
<tr>
<td>Stable fragment</td>
<td>0.27</td>
<td>4.0</td>
<td>10,000</td>
</tr>
</tbody>
</table>

Parent Protein is Not Susceptible to Cleavage by Pepsin

The enzymatic activity of Pepsin, shown in Figure 10, is unique compared to proteolysis of NCAD12 by α-Chymotrypsin and Endoproteinase Glu-C. Initially, we
expected that lowering the pH would result in high proteolysis of NCAD12 by Pepsin. However, it appears that only the contaminant band is being cleaved. This is evident because the full-length parent band does not decrease in abundance despite the increase in exposure time, but the contaminant bands do decrease as exposure time increased. We compiled several hypotheses to explain this phenomenon. One possible explanation is that the lower pH resulted in increased stability of this acidic protein. Due to the decreased pH, the side chains of NCAD12’s many acidic amino acid residues would become protonated, thus neutralizing their charges. The neutralization of these charges might stabilize the protein to such an extent that it is no longer susceptible to cleavage by Pepsin. The peptide contaminant must be susceptible to cleavage by Pepsin because its abundance decreases as exposure time to pepsin increases. Additionally, the absence of NCAD12’s many negative charges would result in a decrease in affinity for calcium ions. The loss of calcium binding would explain why the absence of cleavage is uniform despite the varying calcium concentrations.

It is also possible that the Pepsin was inactive. We performed experiments with a small chromogenic substrate N-Succinyl-Ala-Ala-Pro-Phe-p-Nitroanilide (AAPF; Sigma Aldrich) at acidic pH. Preliminary results showed that our Pepsin stock was active (data not shown). This independent assay of enzyme activity is under development in the lab at this time. Preliminarily, this independent test of Pepsin activity along with the cleavage of the contaminating peptide bands supports that the enzyme was active.

The most plausible explanation for these surprising results is that cadherin precipitated out of solution. This would result from the changes in structure that occurred with such a drastic change in its environment to a pH of 3.7, thus causing the protein
molecules to form aggregates. If cadherin precipitated out of solution, then pepsin, which is in solution, would no longer be able to cleave it. However, cadherin would still be present on the SDS-PAGE gel. This hypothesis explains why the parent bands are not decreasing in abundance and why there are no differences between the varying calcium concentrations.

**Figure 10.** Cleavage of NCAD12 by Pepsin as a Function of pH. Lane 1 ultra low molecular weight standard, lane 2 Apo conditions at 0 min., lane 3 Apo conditions at 30 min., lane 4 1,000 µM calcium conditions at 0 min., lane 5 1,000 µM calcium condition at 30 min., lane 6 100 µM calcium conditions at 0 min, lane 7 100 µM calcium conditions at 30 min, lane 8 10 µM calcium conditions at 0 min., lane 9 10 µM calcium conditions at 30 min. Cleavage of NCAD12 by Pepsin does not occur. However, cleavage of the contaminant band does occur as exposure time increases. The inability of Pepsin to cleave NCAD12 is most likely the result of increased stability of NCAD12 due to protonation of side chains of acidic amino acids at the low pH. The loss of negative charges may stabilize the protein to an extent that it is no longer susceptible to cleavage by Pepsin.

**Endoproteinase Glu-C**

**Enzyme Activity is Independent of pH**

Enzymatic activity of Endoproteinase Glu-C was initially tested in Apo conditions in order to test the proteinase’s dependence upon pH (Figure 11). Samples were taken at 0 minutes and after incubation overnight for approximately 18 hours. A longer
incubation time of 2-18 hours is required for cleavage by Endoproteinase Glu-C. Because the doublet is once again present in the control lanes, its presence can be overlooked (lanes 4, 7, and 10). Through comparing the bands formed in the overnight lanes (lanes 3, 6, and 9) to the 0 minutes lanes (lanes 2, 5, and 8), it can be concluded that the extended incubation period allowed for cleavage of the full-length parent band into a stable fragment. Thus, regardless of the pH condition, the same peptide fragment was formed. Notice that in each overnight sample there is a remarkably similar density of the full length and stable fragment bands, despite the differences in pH. Therefore, these results demonstrate that the enzymatic activity of Endoproteinase Glu-C is not dependent upon pH in the range of pHs tested. This is an important control for further studies, as it proves that any changes in protein cleavage is the result of other factors being manipulated. Other gels of this study are located in the Appendix.

Figure 11. Cleavage of NCAD12 by Endoproteinase Glu-C as a Function of pH. Lane 1 ultra low molecular weight standard, lane 2 pH 6.0 at 0 min., lane 3 pH 6.0 overnight, lane 4 pH 6.0 control, lane 5 pH 7.0 at 0 min., lane 6 pH 7.0 overnight, lane 7 pH 7.0 control, lane 8 pH 7.4 0 min., lane 9 pH 7.4 overnight, lane 10 pH 7.4 control. The remarkably similar bands in the lanes containing overnight samples are indicative that the enzymatic activity of Endoproteinase Glu-C is independent of pH.
Determination of the Size of the Stable NCAD12 Fragment

The estimated size of the stable fragment is 9,950 D. Because of our interest and the importance of the identity of this stable fragment, we made a peptide map to determine possible cleavage sites that would result in a probable identity of the fragment (Figure 12). Endoproteinase Glu-C cleaves at one of the nine glutamates that are contained within N-cadherin. After determining the length of all the possible fragments produced, including primary and secondary cleavage products, six possible peptides were identified as possible candidates, which are shown in Figure 11. Based on our knowledge of the high susceptibility of EC2 to proteolysis by α-Chymotrypsin, we expect that the stable peptide fragment will be contained within EC1. Beyond the exclusion of the three EC2 peptides, we are not capable of knowing which of the other three peptide fragments it might be.

![Figure 12. Estimation of Size of Stable Fragment. Peptide map of all the possible NCAD12 fragments that are similar to the predicted MW that would be formed by cleavage with Endoproteinase Glu-C. The predicted MW of the stable fragment was found using Rf values calculated by the migration of the fragments in the SDS-PAGE gel.](image)
**Calcium Binding Affinity Decreases with pH**

Because the activity of Endoproteinase Glu-C is independent of pH, it was the proteinase used to detect the difference in cleavage of NCAD12 with the manipulation of calcium concentration. Our hypothesis is that as pH is lowered, NCAD12 will have a lower affinity for calcium, which would make this protein more susceptible to cleavage. Therefore at a lower pH more cleavage should be seen. The gel shown in Figure 13 demonstrates this occurrence. Once again, the overnight lanes at pH 6.0 and 7.0 demonstrate the formation of a stable fragment band. However, there is no stable fragment apparent in the overnight sample at pH 7.4 (lane 9). In comparison to the Apo gel (Figure 11) overnight sample at pH 7.4 (lane 9), there is cleavage of the full-length parent band and a notable fragment band. Therefore, it can be concluded that an addition of calcium caused a decrease in cleavage. Also notable is the decrease in intensity of the fragment band in the overnight lanes as the pH increases. Thus, the lower pH in lane 3, with a pH of 6.0, causes NCAD12 to have a lower affinity for calcium and become more susceptible to cleavage in comparison to NCAD12 in lane 6, with a pH of 7.0. Therefore, this gel not only demonstrates the protective effect calcium has for cadherin, but it also demonstrates the decreasing effect pH has on NCAD12’s affinity for calcium, thus allowing for increased cleavage by Endoproteinase Glu-C. Other gels of this study are located in the Appendix.
Figure 13. Cleavage of NCAD12 by Endoproteinase Glu-C as a Function of 10 µM Calcium Concentration and pH. Lane 1 ultra low molecular weight standard, lane 2 pH 6.0 at 0 min., lane 3 pH 6.0 overnight, lane 4 pH 6.0 control, lane 5 pH 7.0 at 0 min., lane 6 pH 7.0 overnight, lane 7 pH 7.0 control, lane 8 pH 7.4 at 0 min., lane 9 pH 7.4 overnight, lane 10 pH 7.4 control. In comparison to the Apo gel, this gel depicts decreased cleavage of NCAD with the addition of calcium. Also, the disparity in the density of the stable fragment bands in the overnight lanes is clear evidence that pH does matter in NCAD’s binding affinity for calcium.

pH Increases and pCa Decreases Susceptibility to Endoproteinase Glu-C

The combined effects of pH and calcium concentration on proteolysis of NCAD12 were tested using Endoproteinase Glu-C (Figure 14). Endoproteinase Glu-C’s proteolytic activity is independent of pH, therefore, any changes in proteolysis as a result of pH are due to the manipulation of calcium and its effect on NCAD12’s susceptibility to cleavage.

First, consider the time dependence of proteolysis. In all three gels, we were surprised to find some cleavage at 0 minutes. This is evident when comparing the density of the bands at 0 minutes (lanes 2, 3, and 4) with the bands in the control (lane 5). Cleavage greatly increased as exposure time increased, which can be easily noted when comparing the 0 minute lanes to the lanes that were incubated overnight for 18 hours.
Second, consider calcium dependence of proteolysis. Protection from cleavage can be seen as the calcium concentrations are increased. A higher calcium concentration of 100 µM resulted in more protection of NCAD12 and thus less cleavage of the parent band into the stable fragment than did the 10 µM calcium concentration. Comparison of the high and low calcium concentrations at both 0 minutes and after overnight incubation show the progressive decrease in cleavage with the addition of a higher calcium concentration. This trend is evident in Figure 14A and Figure 14B, but Figure 14C does not appear to follow this trend. The exception present at the lower pH will be discussed below.

Third, consider the pH dependence of proteolysis. Overall there is more cleavage of cadherin in the low pH condition of pH 6.0 (Figure 14C). This is interesting and can be justified in several ways. It implies that overall there is less calcium binding to cadherin at pH 6.0 leading to an increased susceptibility to proteolysis. It also could be due to a change in the cleavage specificity of the enzyme such that aspartates become a better substrate for the protease leading to an increase in the number of fragments formed. The density of the stable fragment band at pHs 7.4 and 7.0 look very similar (Figure 14A, 14B).
Figure 14A. Cleavage of NCAD12 by Endoproteinase Glu-C as a Function of Calcium Concentration at pH 7.4. Lane 1 ultra low molecular weight standard, lane 2 Apo conditions at 0 min., lane 3 low calcium conditions at 0 min., lane 4 high calcium conditions at 0 min., lane 5 control (Apo) at 0 min., lane 6 Apo conditions overnight, lane 7 low calcium conditions overnight, lane 8 high calcium conditions overnight, lane 9 control (high calcium).

Figure 14B. Cleavage of NCAD12 by Endoproteinase Glu-C as a Function of Calcium Concentration at pH 7.0. Lane 1 ultra low molecular weight standard, lane 2 Apo conditions at 0 min., lane 3 low calcium conditions at 0 min., lane 4 high calcium conditions at 0 min., lane 5 control (Apo) at 0 min., lane 6 Apo conditions overnight, lane 7 low calcium conditions overnight, lane 8 high calcium conditions overnight, lane 9 control (high calcium).
Figure 14C. Cleavage of NCAD12 by Endoproteinase Glu-C as a Function of Calcium Concentration at pH 6.0. Lane 1 ultra low molecular weight standard, lane 2 Apo conditions at 0 min., lane 3 low calcium conditions at 0 min., lane 4 high calcium conditions at 0 min., lane 5 control (Apo) at 0 min., lane 6 Apo conditions overnight, lane 7 low calcium conditions overnight, lane 8 high calcium conditions overnight, lane 9 control (high calcium).

Using QuantityOne software, a method was developed to quantitatively assess for the difference in cleavage due to an increase in calcium concentration. As can be seen at the top of Figure 15, contrast tools show the difference in densities of the stable fragment band as the calcium concentration increases. The bar depicting the density of the Apo band is much higher than the bar depicting the density of the 10 µM Ca²⁺ band, which is much higher than the bar depicting the density of the 100 µM Ca²⁺ band. The contrast tools available through QuantityOne Software enabled for an increased understanding and portrayal of the trend of decreased cleavage with increased calcium concentration.
Figure 15. Demonstration of How Density Values were Acquired. Using QuantityOne software, the same software used to visualize and photograph gels, density values were acquired through contrast tools. The background of each lane was recorded and subtracted from the values for each band. This allowed for an objective qualitative comparison of bands in the same and in different gels. Additionally, it was especially useful in quantitatively demonstrating trends in cleavage by Endoproteinase Glu-C due to the manipulation of calcium concentration and pH.
DISCUSSION

Due to the essential role of cadherins as the primary calcium-dependent cell-cell adhesion molecule, the integrity and maintenance of their structure is of great importance in creating endothelial and epithelial barriers and functioning neuronal synapses. Normally, cadherin is resistant to cleavage by proteases due to stabilization of the protein structure by calcium. Two main environmental factors that contribute to the ability of proteases to degrade cadherins are dysregulated calcium concentration and pH. The concentration of calcium affects cadherin because, as it binds, it exhibits a protective effect for the protein through stabilization of its structure. Therefore, decreased calcium concentration should result in increased cleavage. When pH is lowered it decreases the binding affinity of cadherins for calcium by protonating the acidic carboxylates in the calcium binding sites. The dysregulation of these factors result in a decrease in the integrity of the structure of cadherin, and a diseased state can follow. The focus here is on the entry of *Candida albicans* through the use of SAPs to cause candidiasis and the oncogenetic effects of cancer. Therefore, a proteolytic method was established to assess the effect of the pH-dependence and the pCa-dependence on the susceptibility of NCAD to cleavage by various proteinases. The results of this study show, as predicted, that a decrease in pH and an increase in pCa result in an increase in proteolytic susceptibility of NCAD12.
The three proteinases used vary in their specificity. α-Chymotrypsin, a serine protease, preferentially cleaves on the C-terminal side of the aromatic amino acids tyrosine (Y), phenylalanine (F), and tryptophan (W). Additionally, α-Chymotrypsin is active within a wide range of pH but has an optimum pH of 7.8. Pepsin, on the other hand, cleaves nonspecifically. However, at acidic pH, Pepsin shows a preference for cleaving bulky hydrophobic amino acids. Pepsin was chosen because it is a member of the SAP family and because it is active at very low pH range of 2.0-4.0. The protease Endoproteinase Glu-C, a serine protease, shows specificity for cleaving peptide bonds on the C-terminal side of glutamate and aspartate residues, however cleavage of aspartate is exceptionally rare in such an acidic protein. It is also active over a broad range of pH from 3.5 to 9.5, but exhibits maximal activity between 4.0 and 7.8. Endoproteinase Glu-C was chosen because its range of optimum pH coincides with the range of pH being investigated. It required a longer incubation time of 2-18 hours. However, despite the differences in cleavage specificity between these various proteases, there is persistent cleavage of a stable fragment. Calculations performed using Rf values consistently predicted an experimental molecular weight close to 10,000 g/mol.

Furthermore, each of the various proteases used reveal some information about the effect of pH on cadherin. Experiments performed using α-Chymotrypsin show that increased exposure time results in increased cleavage of NCAD12 (Figure 8). Also, the Rf calculations were performed on the stable fragment produced by α-Chymotrypsin and the results showed that the fragment has a molecular weight around 10,000 g/mol (Table 5). A peptide map was created and utilized to discover the possible cleavage positions and the possible identity of the stable fragment. Discussion of α-Chymotrypsin’s stable
fragment in comparison to Endoproteinase Glu-C’s stable fragment will be discussed below.

Experiments performed using Pepsin demonstrated little to no cleavage of NCAD12. This experiment was interesting because it was not the result that we were expecting. The fact that Figure 10 shows that NCAD12 displayed little to no susceptibility to Pepsin, in the range of its optimum pH, is very intriguing. A possible explanation is that the drastic decrease in pH to 3.7 protonated the side chains of many of NCAD12’s many acidic amino acid residues, thus neutralizing the electrostatic repulsion and resulting in stabilization of the protein. The changes in electrostatic repulsion might stabilize the protein to such an extent that it is no longer susceptible to cleavage by Pepsin with or without calcium bound. Thermal denaturation experiments underway in our lab support a slight increase in stability of the Apo state of NCAD12 as the pH decreases from pH 7.4 to pH 6.0 (data not shown). The chromogenic substrate assay for Pepsin activity must be developed in order to eliminate a trivial cause for these data. Additionally, precautions must be taken in order to prevent cadherin from precipitating out of solution when lowering the pH.

Experiments performed using Endoproteinase Glu-C illustrated the relevance of many factors that are correlated with the stability of NCAD12 and its cleavage. First, it was discovered that Endoproteinase Glu-C’s proteolytic activity is independent of pH and that increased exposure time results in increased cleavage of NCAD12 (Figure 11). Therefore, Endoproteinase Glu-C was used to test the effects of pCa and pH on the proteolytic susceptibility NCAD12. The results show that an increase in the concentration of calcium results in an increase in protection from cleavage (Figure 13,
In other words, the higher the calcium concentration the less cleavage of the full-length parent band seen. This is in accordance with our hypothesis that calcium protects cadherin from cleavage due to its binding stabilizing cadherin’s structure. The results also demonstrate that a decrease in pH to 6.0 increases the amount of overall cleavage of NCAD12 in comparison to the cleavage at pH 7.0 and 7.4 (Figure 14). This is also the result expected because as pH decreases, cadherin’s affinity for calcium also decreases, thus resulting in increased susceptibility to cleavage.

Additionally, Rf calculations were performed on the stable fragment produced by Endoproteinase Glu-C and the results showed that the fragment has a molecular weight of close to 10,000 g/mol (Figure 12). A peptide map was created and utilized to discover the possible cleavage positions and the possible identity of the stable fragment. The fact that this stable fragment has a similar molecular weight to the stable fragment produced by α-Chymotrypsin is revealing of the susceptibility of NCAD12 to cleavage of any type. α-Chymotrypsin and Endoproteinase Glu-C both possess different proteolytic specificity but the stable fragment produced is similar. Therefore, it can be deduced that NCAD12 is more susceptible to cleavage in some positions of its structure than others. The Rf calculations reveal that the stable fragment is approximately the size of one an EC domain. It is known that EC1 is significantly more stable than EC2. This has been demonstrated through EC1 displaying a 20°C increase in the denaturation temperature ($T_m$) over the $T_m$ of EC2 at neutral pH (50). Therefore, it can be deduced that the stable fragment produced is likely to be a fragment consisting of the EC1 domain.

Overall, these studies reveal that pH is a factor contributing to differential cleavage of NCAD12. Specifically, it has shown that a decrease in pH results in an
increase in cleavage by Endoproteinase Glu-C. The most plausible explanation for this occurrence is that the decrease in pH causes a decrease in NCAD12’s binding affinity for calcium, which exhibits a protective effect for the protein. Although the range of pH from 6.0 to 7.4 is a limited range, it is of physiological importance in the diseases that are relevant to cadherin, such as candidiasis and cancer. Furthermore, the subtle change of cleavage seen over this narrow range is in the direction that was expected. Therefore, these data contribute to a growing series of studies that each support that stability and calcium binding of N-cadherin is affected by subtle changes in its environment.
Future studies

Review of the literature highlights the utility of developing methods for studying the effect of subtle pH changes on the structure and function of cadherin. Regulation of pH and calcium levels in vivo is essential for maintenance of cell adhesion. This is somewhat problematic since proton and calcium concentrations in body cavities are not easily regulated. Since the first contact point of pathogens like *Candida albicans* is with the epithelial barrier tissues, the environment of the lumen and its effect on pathogenicity is an important issue. Dr. Pedigo’s lab has a long term interest in cadherin-mediated virulence mechanisms. Work continues on expressing and purifying SAP5 to use for future experiments. Additionally, Dr. Pedigo’s lab will determine the exact size of the stable fragment using mass spectrometry.

In the realm of cancer research, the most important question is whether or not dysregulated pH is necessary for normal differentiated human cells to become malignant. Once this question has been addressed, there are multiple areas of research that could lead to innovative therapeutic agents and processes to treat cancer. Because the dysregulated pH of cancerous cells is a common adaptive feature of most cancers, regardless of genetic background or tissue origin, physiological pH sensors promise to be increasingly important as therapeutic targets in order to increase specificity and efficacy (47). By targeting these specific physiological pH sensors, doctors would be better able to treat cancers with a more specific approach depending on the pH sensor being affected by the cancer. The effects of such treatment would help prevent the cells from evading apoptosis and becoming highly prolific. Additionally, the lower extracellular pH could be exploited to promote site-specific drug delivery in innovative ways such as micelles.
Research contributing to the understanding of how an increased intracellular pH and a decreased extracellular pH lead to proliferation, survival and invasion has the potential to lead to development of new targets for cancer therapy that could span between different classes of cancers (47).
LIST OF REFERENCES


APPENDIX
Figure 16. Cleavage of NCAD12 by Endoproteinase Glu-C in Apo Conditions. Lane 1 pH 7.0 overnight, lane 2 pH 7.4 control, lane 3 pH 7.4 overnight, lane 4 pH 7.4 at 20 min., lane 5 pH 7.4 at 0 min., lane 6 pH 6.0 control, lane 7 pH 6.0 overnight, lane 8 pH 6.0 at 20 min., lane 9 pH 6.0 at 0 min., lane 10 ultra low molecular weight standard.

Figure 17. Addition of 1,000 µM Ca^{2+} Inhibits Cleavage. Lane 1 ultra low molecular weight standard, lane 2 pH 6.0 at 0 min., lane 3 pH 6.0 overnight, lane 4 pH 6.0 control, lane 5 pH 7.0 at 0 min., lane 6 pH 7.0 overnight, lane 7 pH 7.0 control, lane 8 pH 7.4 at 0 min., lane 9 pH 7.4 overnight, lane 10 pH 7.4 control.
Figure 18. Addition of 100 µM Ca\(^{2+}\) and Cleavage of NCAD12. Lane 1 pH 6.0 at 0 min., lane 2 pH 6.0 overnight, lane 3 pH 6.0 control, lane 4 pH 7.0 at 0 min., lane 5 pH 7.0 overnight, lane 6 pH 7.0 control, lane 7 pH 7.4 at 0 min., lane 8 pH 7.4 overnight, lane 9 pH 7.4 control, lane 10 ultra low molecular weight standard.