Characterization of the specificity of human neutrophil elastase for *Shigella flexneri* virulence factors

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Zusammenfassung

Neutrophile Granulocyten wirken als einer der ersten Abwehrmechanismen gegen invasive Mikroorganismen im angeborenen Immunsystem von Mammalia. Aktiviert durch inflammatorische Signale verlassen diese Granulocyten das vaskuläre System und migrieren durch das Gewebe zum Infektionsherd. Dort binden sie die Mikroorganismen, phagozytieren und eliminieren diese schließlich mit hoher Effizienz. Humane Neutrophile Elastase (NE) ist Bestandteil der neutrophilen Granula und spielt eine entscheidende Rolle im Abbau von Virulenzfaktoren enteroinvasiver Bakterien, einschließlich der *Shigella* Virulenzfaktoren IpaB (invasion antigen plasmid B) und IcsA (intracellular spread A).

Der Grund für die spezifische Aktivität von NE gegenüber diesen Faktoren ist bisher nicht bekannt. Unsere Analyse impliziert, dass die Primärstruktur von IpaB keine Rolle für die Spezifität von NE spielt. Eine Reihe von IpaB Mutanten, welche Deletionen u.a. in der coiled-coil Region sowie der möglichen Transmembrandomänen enthielten, wurden von NE genauso abgebaut wie wildtyp IpaB. Des Weiteren scheint auch die Sekundär- und Tertiärstruktur des Substrats nicht ausschlaggebend für die Erkennung durch NE zu sein, da denaturiertes IpaB ebenfalls von NE abgebaut wurde.

NE gehört zu der Familie der Chymotrypsin-ähnlichen Serinproteasen, die sich durch Sequenz- und Strukurähnlichkeit auszeichnen, jedoch sehr unterschiedliche biologische Funktionen aufweisen. Cathepsin G (CG) ist wie NE eine Chymotrypsin-ähnliche Serinprotease und ebenfalls in neutrophilen Granula lokalisiert. Allerdings zeigt CG keine Aktivität gegenüber Virulenzfaktoren von *Shigella*. Obwohl die Kristallstrukturen von CG und NE fast identisch sind, konnten einzelne oder mehrere Aminosäuren in der Substratbindungsspalte identifiziert werden, die zwischen den beiden Enzymen differieren. Dies legte die Vermutung nahe, dass die Spezifität von NE gegenüber Virulenzfaktoren in diesen Unterschieden codiert sein könnte. Daher wurden diese Aminosäuren durch die analogen CG Aminosäuren oder durch Alanin ersetzt. Der Vergleich der funktionellen Eigenschaften der NE Mutanten mit wildtyp NE zeigte, dass die Aminosäuren an den Positionen 98 und 216-224 entscheidend für die Substratspezifität von NE sind. Die NE Mutanten N98A, 216-218 und 216-224 waren nicht mehr in der Lage, die Virulenzfaktoren IcsA und IpaB sowie das NE Peptidsubstrat abzubauen. Stattdessen haben diese Mutanten die Fähigkeit erlangt, das CG Peptidsubstrat abzubauen. Zusammenfassend konnten wir Aminosäuren in NE identifizieren, die sowohl die Spezifität von NE für das Peptidsubstrat als auch für die Virulenzfaktoren von *Shigella flexneri* determinieren.

Schlagworte: Neutrophile Elastase, *Shigella flexneri*, Spezifität, Virulenzfaktoren

Abstract

Neutrophil granulocytes are one of the first lines of defense of the mammalian innate immune system against invading microorganisms. In response to inflammatory stimuli, neutrophils migrate from the blood stream to infected tissues where they bind, engulf and inactivate microorganisms efficiently. Human neutrophil elastase (NE), a neutrophil granule component, is a key host defense protein that rapidly destroys virulence factors of enteroinvasive pathogens including IpaB (invasion plasmid antigen B) and IcsA (intracellular spread A) from *Shigella*.

The structural basis of the exquisite sensitivity of virulence proteins to NE is not known. Our analysis suggests that the primary structure of IpaB is not important for NE specificity. Using a series of IpaB mutants that contained deletions in the coiled-coil region, as well as in the putative transmembrane domains of the hydrophobic region, we observed that the susceptibility of the IpaB mutants to NE was similar to that of the wildtype IpaB. Secondary or tertiary structures of the substrate are also unlikely to play a role in the recognition of virulence factors by NE since heat-denatured and native IpaB were equally well targeted by NE.

NE belongs to the family of chymotrypsin-like serine proteases with sequence and structural similarity but with very different biological functions. Cathepsin G (CG) is another abundant chymotrypsin-like serine protease in neutrophil granules. However, in contrast to NE, CG does not cleave virulence factors of *Shigella*. The crystallographic structures of NE and CG are very similar but we identified single or multiple residues in the substrate-binding cleft to differ in these two enzymes. We hypothesized that NE specificity for bacterial virulence factors resides within these structural differences. Therefore these specific residues in NE were replaced with the analogous amino acids of CG or with alanine. By comparing the functional properties of these NE mutants to wildtype NE we were able to show that the amino acids at position 98 and 216-224 are crucial for the substrate specificity of NE. The NE mutants N98A, 216-218 and 216-224 did not cleave the virulence factors IcsA and IpaB as well as the NE peptide substrate but cleaved the CG peptide substrate. In summary, we identified residues in NE that determine the specificity of NE for the peptide substrate and for the *Shigella flexneri* virulence factors.

Keywords: neutrophil elastase, *Shigella flexneri*, specificity, virulence factors

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1 Introduction

Humans are constantly exposed to myriads of microorganisms. Some are harmless transients, others become part of the commensal flora that we harbor for a lifetime. Numerically, we carry more microbial cells than we have cells of our own. We would not survive long without our constant "normal" microbial flora. Yet we are in a delicate balance with these microorganisms. If our innate immunity is compromised, the harmless microorganisms can quickly become serious or even fatal threats to our health. In addition, among the microorganisms that humans encounter each day are those whose survival depends on their ability to cause cellular damage to their host. This group of microorganisms is called pathogens. A pathogen must enter a host and multiply sufficiently to establish itself or to be transmitted to a new susceptible host. Pathogens damage their host by intoxication or compromising the integrity of the cells. In most cases the pathogen-induced damage is not serious but a proportion of hosts will suffer from disease or even be killed. A common group of disease causing pathogens are enterovasive bacteria. One of them is *Shigella.*

1.1 *Shigella*

1.1.1 Epidemiology

Shigellae are enteropathogenic bacteria that cause the diarrhea-disease dysentery. In the environment *Shigella* can be found in brackwater but their only natural hosts are primates. Humans take up *Shigella* from stools or soiled fingers of infected persons or from contaminated food and water. Shigellosis is characterized by mucous diarrhea, fever, nausea and stomach cramps. All *Shigellae* species are able to cause bacterial dysentery, in which the diarrhea is not only mucopurulent but also bloody (Sansonetti 1992). *Shigella* is extremely efficient in invading the hosts' intestinal epithelium and causing disease: 10-100 bacteria are sufficient to cause shigellosis (DuPont et al. 1989). Symptoms occur one to two days after exposure to the bacteria. The host response usually leads to the resolution of the infection within five to seven days. However, without proper medical treatment the diarrhea can be life threatening to some persons, especially young children and the elderly. This treatment involves rehydration as well as application of electrolytes and antibiotics.

Shigellosis occurs throughout the world with approximately 164.7 million cases per year. Among those cases 99% of them are found in areas of the world with only limited medical support and poor sanitation causing low hygienic standards. Each year 1.1 million people are estimated to die from *Shigella* infection (Kotloff et al. 1999). 61% of all fatalities attributable to shigellosis involve children less than 5 years of age as systemic complications of shigellosis occur frequently in children (CDC 2005; WHO 2005). These complications include acute renal failure, hemolytic-uremic syndrome, toxic megacolon and neurological sequelae (Goldfarb et al. 1982).

The *Shigella* genus comprises four species: *S. dysenterie, S. boydii, S. flexneri* and *S. sonnei*. This order reflects the severity of symptoms (Mims C. 1998). *S. sonnei* is classically found in developed countries (Kotloff et al. 1999). But it seems to become more prevelant in Thailand (71%) as compared to previous years, a phenomenon probably linked to the current development of the country. *S. flexneri* is predominant in developing countries (60%) and it is the most frequently isolated species worldwide. *S. dysenteriae* type 1 (Sd1) is the only *Shigella* species causing epidemic dysentery. Epidemic outbreaks have occurred throughout the world but are often linked to confined populations, e.g. refugee camps. Approximately 5-15% of Sd1 cases are fatal since Sd1 is resistant to many antimicrobials. *S. dysenteriae* is also the only *Shigella* species producing the Shiga toxin. This toxin can translocate into eukaryotic target cells and inhibits protein synthesis (WHO 2005).

1.1.2 Properties

Shigella was first described 1897 by Shiga Kiyoshi in Japan. *Shigellae* are rod-shaped, non-motile Gram-negative bacteria. They belong to the family of enterobacteriaceae and do not form spores. *Shigella* is closely related to *Escherichia* and is occasionally considered as one strain of the *E. coli* species. In fact, *Shigella* shares morphological features with *E. coli* but it can be easily distinguished biochemically. For example, the cell wall antigens, also known as O-antigens, of *Shigella* and *E.coli* are distinct. The O-antigen is the outer polysaccharide portion of the lipopolysaccharide (LPS) and consists of repeating sugar units. Furthermore *Shigella* is anaerogenic, meaning it does not produce gas from carbohydrates, and it cannot ferment lactose (School 1995; Levinson W. 2002).

The disease causing properties of *Shigella* are encoded on a virulence plasmid. Strains cured of this plasmid are non-pathogenic (Sansonetti et al. 1982). If the *Shigella* virulence plasmid

is transferred into a non-pathogenic *E. coli*, the plasmid confers invasiveness and cytotoxicity *in vitro* (Sansonetti et al. 1983).

1.1.3 Pathogenicity

After passing the oesopharynx, stomach and small intestine *Shigella* invades the hosts' large intestinal epithelium [figure 1.1 and (LaBrec 1964)]. *Shigella* traverses the epithelial barrier through specialized membranous epithelial cells, called M-cells (Wassef et al. 1989). M-cells transport antigens, including enteric pathogens, across the epithelium. They are located in the epithelium covering the gut-associated lymphoid tissue (GALT) (Kraehenbuhl and Neutra 1992). M-cells are the only port of entry across the epithelium for *Shigella*, because *Shigella* cannot invade colonocytes through their apical membrane (Mounier et al. 1992). Following passage across M-cells, the microorganism interacts with two different host cells: epithelial cells and macrophages. *Shigella* invades the epithelial cells from the basolateral side. It escapes from the phagosome to the cytoplasm of the cells and replicates there. In order to move intra- and intercellularly, *Shigella* utilizes the cytoskeleton of the host cells (Makino et al. 1986). Once infected, the cells secrete the cytokine interleukin-8 (IL-8) to recruit neutrophils (also called polymorphonuclear leukocyte, PMNs) to the site of infection. The other host cells *Shigella* encounters are resident tissue macrophages. They are situated within lymphoid follicles beneath M-cells (Jarry et al. 1989; Soesatyo et al. 1990). Although the macrophages phagocytose *Shigella,* the bacteria can escape from the phagolysosome to the cytoplasm within minutes (Finlay and Falkow 1988; Maurelli and Sansonetti 1988). Unlike in the epithelial cells, *Shigella* rapidly induces macrophage apoptosis (Zychlinsky et al. 1992). In an apoptotic process, a cell synthesizes the molecules responsible for its own death (Arends and Wyllie 1991). Accordingly, macrophages infected with *Shigella* show the two cardinal signs of apoptosis: specific morphological changes and fragmentation of nuclear DNA into multimers of approximately 200 bp.

Shigella escapes from the dying macrophage and infects further epithelial cells. The apoptotic macrophage releases the pro-inflammatory cytokines interleukin-1β and –18 (IL-1β and IL-18) to recruit neutrophils to the site of infection. Although counterintuitive, the neutrophils first support the bacterial infection. They damage the colonic mucosa by breaking tight junctions to reach bacteria in the intestinal lumen. Thus they promote the entry for *Shigella* into the epithelium. But eventually neutrophils resolve the infection.

Neutrophils engulf *Shigella* but in contrast to macrophages they prevent the escape of *Shigella* from the phagolysosome and kill them.

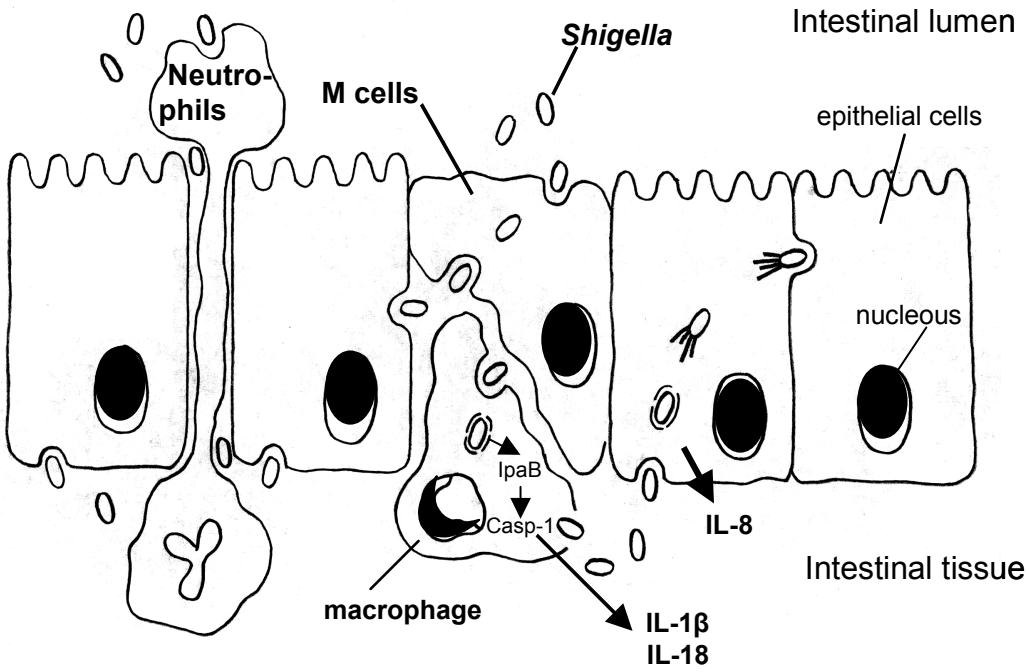


Fig. 1.1: *Shigella* infection.
Shigella invades the epithelium from the intestinal lumen through M-cells. After reaching the epithelium it invades epithelial cells and is phagocytosed by resident macrophages. *Shigella* escapes the phagosome of both cells but while *Shigella* replicates within epithelial cells it induces apoptosis in macrophages probably by activation of caspase-1 (Casp-1). The dying macrophages release the pro-inflammatory cytokines IL-1β and IL-18. Together with IL-8 secreted from the invaded epithelial cells, they signal for PMN (polymorphonuclear leukocyte or neutrophils). The recruited neutrophils eventually clear the infection.

1.1.4 Virulence Factors

The degree to which pathogenic bacteria are able to cause disease determines their virulence. Virulence depends on the resistance of the host and as well as on the invasiveness and toxicity of the bacteria. Bacterial components and proteins that mediate adhesion, invasion, toxicity, and evasion of host immune cells are termed virulence factors (Mayer-Scholl et al. 2004). *Shigella* virulence factors are encoded on a 220 kb virulence plasmid which is essential for *Shigella* pathogenicity (Sansonetti et al. 1982). *S. flexneri* invasion genes are localized in a 31 kb region of the virulence plasmid (Maurelli et al. 1985). This region encodes the invasion plasmid antigens (ipa) operon, the membrane expression of ipas (mxi) and surface presentation of invasion plasmid antigens (spa) operons, as well as other,

independently expressed genes. By transposon insertion and deletion mutagenesis, *ipaB*, *ipaC* and *ipaD* genes were shown to be essential for invasion, vacuolar escape, induction of macrophage apoptosis and virulence in animal models (Sasakawa et al. 1988; High et al. 1992; Menard et al. 1993). IpaB, C and D are secreted by a type III secretion apparatus encoded by the mxi and spa operons (Andrews et al. 1991; Allaoui et al. 1992; Allaoui et al. 1993). The type III secretion apparatus is composed of at least 30 proteins and is conserved among enteropathogenic bacteria (Cornelis and Van Gijsegem 2000). IpaB and IpaC form a complex (Menard et al. 1994) that appears to be sufficient to invade epithelial cells (Menard et al. 1996).

In addition to its role in invasion, several lines of evidence indicate that the *Shigella* virulence factor IpaB is both necessary and sufficient to induce apoptosis in macrophages. First, mutant strains of *Shigella* that are invasive but do not express IpaB are not cytotoxic (Zychlinsky et al. 1994). Second, microinjection of IpaB into the cytosol of macrophages efficiently triggers apoptosis (Chen et al. 1996). Third, IpaB was shown to bind caspase-1 [also called IL-1β converting enzyme (ICE)] which plays an important role in *Shigella* induced apoptosis (Thirumalai et al. 1997; Hilbi et al. 1998). Caspase-1 is a proapoptotic and proinflammatory cysteine protease. When activated, it cleaves the pro-inflammatory cytokines IL-1β and IL-18 to their biologically active forms (Thornberry 1994; Dinarello 1998). Together these data suggest a model where IpaB is secreted into the macrophage cytosol during infection. IpaB binds to and activates caspase-1, an event that simultaneously induces apoptosis and activates the pro-inflammatory cytokines IL-1β and IL-18.

Another virulence factor encoded on the virulence plasmid is IcsA (intracellular spread, also called VirG). IcsA is essential for intra- and inter-cellular movement of *Shigella* (Bernardini et al. 1989). Disruption of IcsA leads to loss of bacteria induced intracellular actin assembly, loss of cell-to-cell spread, and markedly reduced virulence in humans and animal models (Makino et al. 1986; Bernardini et al. 1989; Lett et al. 1989; Sansonetti et al. 1991; Coster et al. 1999). It is localized to the outer membrane (120 kDa form) and is secreted through a type V system (90 kDa form). IcsA is asymmetrically distributed along the bacterial body (Goldberg et al. 1993). This is a prerequisite for the polar movement of *Shigella* in mammalian cells, including bacterial spreading between epithelial cells. The N-terminal part of the IcsA α domain induces the polymerization of actin by interaction with host proteins such as vinculin and neural Wiskott-Aldrich syndrome protein (N-WASP) (Egile et al. 1999). The non-motile *Shigella* "hooks on" to these actin tails to move through and in between cells.

1.2 Innate immune host cells: neutrophils

The interaction of *Shigella* with the host is usually limited to five to seven days. During this time, *Shigella* primarily challenges two host innate defense cells: macrophages and neutrophils. As described above, macrophages phagocytose *Shigella* but are not capable to retain the bacterium within their phagolysosome and kill it. In fact, macrophages with phagocytosed *Shigella* undergo apoptosis. To resolve the *Shigella* infection, neutrophils need to be recruited to the site of infection. The special neutrophil characteristics enable them to control the *Shigella* infection.

Neutrophils are essential for innate host defense against invading microorganisms, such as bacteria and fungi. Neutrophils are the first cells recruited from the blood stream to sites of infection. They are the most abundant white blood cells (60%) but only have a short half-life if not activated. They are terminally differentiated cells, incapable of cell division, and synthesize very low levels of RNA and protein. Neutrophils are generated from the pluripotent haematopoietic stem cells in the bone marrow and are characterized by multi-lobed nuclei and abundant granules in the cytoplasm, which contain host-defense molecules (Mayer-Scholl et al. 2004).

1.2.1 Neutrophil recruitment

Neutrophils are an essential component of the acute inflammatory response and the resolution of microbial infection. Recruitment to inflamed or infected tissue occurs within minutes to hours. Molecules signaling for neutrophil infiltration are predominantly the aminoterminal formylated methionin bacterial peptide (fMLP), IL-8 and TNF-α from macrophages and epithelial cells, and C5a, C3a and C4a from the complement cascade (Burg and Pillinger 2001). Vasodilatation through TNF-α results in the reduced velocity of blood flow. Physiologically circulating neutrophils in the blood contact the endothelium and transiently interact with it, a phenomenon termed rolling. Molecules mediating this are leucocyte (L), platelet (P) and endothelial (E) selectins, which permit interaction between neutrophils, and neutrophils and endothelial cells (Janeway et al. 2001). After exposure of circulating neutrophils to chemoattractants (IL-8, fMLP, C5a, LTB4), members of the β2-integrin family mediate the conversion of the rolling state to a state of tight stationary adhesion (Burg and Pillinger 2001). Neutrophils adhere to the endothelium and secretory vesicles of the neutrophils are mobilized. Neutrophils transmigrate either between or through endothelial cells. During migration through the tissue neutrophils cleave off or shed

their selectins and proteases are liberated from the different granule subsets degrading vascular basement membranes and the intercellular matrix (Faurschou and Borregaard 2003).

1.2.2 Bacterial recognition by neutrophils

The concept of bacterial recognition is based on so-called pathogen associated molecular patterns (PAMPs), which are recognized by pattern recognition receptors (PRRs) (Gordon 2002). PAMPs are microbial structures, which, upon interaction with elements of the host innate immune system, trigger the initiation of host protective responses accumulating in the clearance of the pathogen by phagocytic cells. PAMPs are ideal targets as they allow distinction between self and microbial non-self. They are found on all microorganisms, which allows a limited number of receptors to recognize PAMPs, and importantly they are essential for microbial survival, therefore no escape mutants can be generated (Mukhopadhyay et al. 2004).

Neutrophils have the following pattern recognition receptors:

- lectins, e.g. dectin-1, which detect bacterial carbohydrates;
- scavenger receptors, e.g. MARCO, are structurally unrelated membrane molecules, which bind and internalize modified lipoproteins, lipopolysaccharides, and lipoteichoic acid;
- complement receptors, e.g. CR1-4, which recognize complement proteins from the serum that have opsonized the microbes;
- Fc receptors, which recognize IgG opsonized microorganisms;
- Toll-like receptors (TLRs) - TLR 2 and 4 are expressed by neutrophils, they recognize lipoproteins and lipopolysaccharide respectively (Muzio et al. 2000);

1.2.3 Neutrophil killing mechanisms

Upon encountering bacteria, neutrophils engulf these microbes into a phagosome, which fuses with intracellular granules to form a phagolysosome (Lee et al. 2003). In the phagolysosome the bacteria are killed after exposure to enzymes, antimicrobial peptides and reactive oxygen species (ROS). The arsenal of cytotoxic agents has been traditionally

divided into either oxygen- independent or -dependent mechanisms (figure 1.2). Both of these systems probably collaborate in killing microbes (Roos and Winterbourn 2002).

The oxygen-independent mechanisms encompass the contents of the three neutrophil granule subsets: the azurophil, specific and gelatinase granules, which contain characteristic proteases, antimicrobial proteins and peptides as well as enzymes (Borregaard and Cowland 1997).

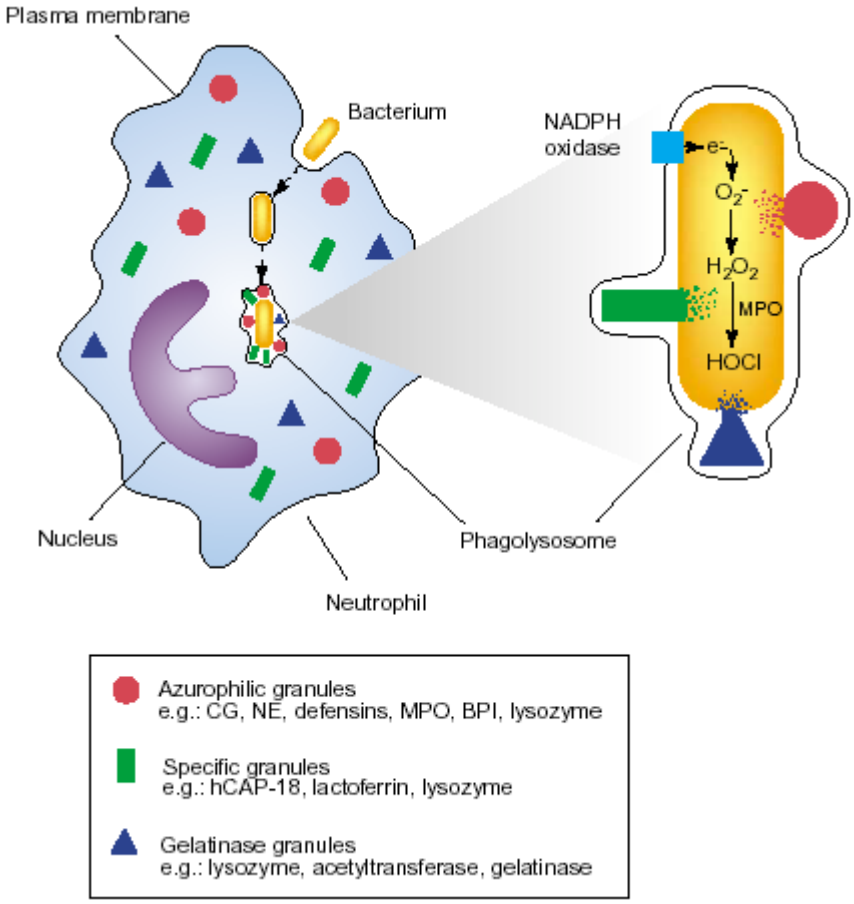


Fig.1.2: Schematic presentation of the oxygen-dependent and oxygen-independent mechanisms during neutrophil phagocytosis of bacteria.
The oxygen-independent mechanisms encompass the contents of the three neutrophil granule subsets: the azurophil, specific and gelatinase granules, which contain characteristic proteases, antimicrobial proteins and peptides, and enzymes. Lysozyme, for instance, disrupts anionic bacterial surfaces, rendering the bacteria more permeable, whereas NE degrades virulence factors. The oxygen-dependent mechanism relies on the NADPH oxidase complex that assembles at the phagosomal membrane and produces O_2^-, which is rapidly converted to hydrogen peroxide. In turn, a constituent of the azurophilic granules, myeloperoxidase, generates hypochlorous acid (HOCl) from hydrogen peroxide. This presentation is taken from (Mayer-Scholl et al. 2004)

Antimicrobial proteins such as defensins, bactericidal/permeability-increasing protein (BPI) and the enzyme lysozyme, predominantly function by disrupting anionic bacterial surfaces, probably rendering the bacteria more permeable (Kagan et al. 1990). Proteases, such as neutrophil elastase (NE), degrade bacterial proteins, including virulence factors (Weinrauch et al. 2002). Other proteases e.g. cathepsin G (CG) have antimicrobial activity independent of their enzymatic activity (Shafer et al. 2002).

The importance of the oxygen-independent mechanism in defense is evident in two very rare inherited diseases, the Chediak-Higashi syndrome (Introne et al. 1999) and Specific Granule Deficiency (Gombart and Koeffler 2002). Both disorders are characterized by recurrent infections and shortened life expectancy. In the Chediak-Higashi syndrome, neutrophils contain giant granules resulting from specific and azurophil granule fusion. Specific Granule Deficiency is characterized by the absence of specific granules and defensins. The severity of the symptoms in these diseases underlines the fundamental role of granule proteins in host defense.

The second mechanism of neutrophil killing is oxygen-dependent (Roos et al. 2003). Phagocytosing neutrophils undergo an 'oxidative burst' during which the NADPH oxidase complex assembles at the phagosomal membrane and produces O_2-, which is rapidly converted to hydrogen peroxide by the enzyme superoxide dismutase. In turn, a constituent of the azurophil granules, myeloperoxidase, generates hypochlorous acid (HOCl) from hydrogen peroxide. How the bacteria are actually killed is not known. Hydrogen peroxide is bactericidal only at high concentrations, therefore a variety of secondary oxidants have been proposed to account for the destructive capacity of the neutrophils (Hampton et al. 1998). The importance of ROS for antimicrobial activity is validated by the susceptibility to infections of patients suffering from chronic granulomatous disease, a condition where the NADPH oxidase complex is inactive (Dinauer et al. 2000).

In the past, studies often focused on the effects of either the oxygen-dependent or oxygen-independent mechanisms. However, a ROS function might also be to recruit K^+ to the phagolysosome, allowing granule proteins to go from a highly organized intra-granule structure into solution (Reeves et al. 2002). The relative contribution of ROS to these two different mechanisms is very intriguing, yet it seems premature to draw conclusions as to whether ROS contribute directly to microbial killing or only serve as activators of granule proteins (Roos and Winterbourn 2002). Besides killing bacteria inside the phagolysosomes, neutrophils can also degranulate and release antimicrobial factors into the extracellular space (Faurschou and Borregaard 2003). The cells can also generate neutrophil extracellular

traps (NETs), which are composed of granule and nuclear constituents that kill bacteria extracellularly (Brinkmann et al. 2004).

1.3 Interaction of neutrophils with *Shigella*

Macrophages phagocytose *Shigella*, but *Shigella* escapes from the phagolysosome of macrophages within minutes. In contrast to that, *Shigella* is trapped within the phagolysosome of neutrophils and is eventually killed. Therefore neutrophils must possess mechanisms to prevent this escape.

Shigella is alive within the phagocytic vacuole of neutrophils for up to one hour (Mandic-Mulec et al. 1997). Thus it is important to retain the bacteria within the vacuole to allow the ROS-dependent and -independent killing mechanisms to exert their functions on the bacterium. One neutrophil protease, neutrophil elastase (NE), seems essential to keep *Shigella* in the vacuole. Neutrophils with pharmacologically inhibited or genetically inactive NE allow the escape of wildtype *Shigella* into the cytoplasm. In these neutrophils, *Shigella* survival rate was also increased (Weinrauch et al. 2002).

How NE exactly contributes to the phagolysosomic retention of *Shigella* was shown in one study that assessed the ability of a human neutrophil granule extract (hNGE) to degrade different *Shigella* virulence factors (Weinrauch et al. 2002). This granule extract was enriched in granule proteases such as NE and CG. Sub-lethal concentrations of hNGE degraded type III secreted virulence proteins IpaA, IpaB and IpaC and the membrane-bound as well as the secreted form of IcsA. The same extract did not affect proteins important for *Shigella* homeostasis such as outer-membrane protein A (OmpA), maltose-binding protein (MBP) or recombinase A (recA), which are outer-membrane, periplasmic and cytosolic proteins, respectively. Using a series of chemical and physiological inhibitors, it was shown that NE was the protease responsible for the observed cleavage of virulence factors. Purified human NE also cleaved IpaB and IcsA but not OmpA at low concentrations (1,2 nM). It was however shown that OmpA from non-pathogenic *E. coli* was degraded by purified NE but only at concentrations that are 2000 times higher than required to cleave virulence factors (Belaaouaj et al. 2000). Purified NE also degrades virulence factors from other Gram-negative pathogens such as *Salmonella* and *Yersinia* (Weinrauch et al. 2002). The apparent specificity of NE for virulence factors is further supported by the fact that NE does not target the type III secretion apparatus itself nor does it cleave secreted *Shigella* proteins not associated with virulence. Interestingly, cathepsin G (CG), another abundant granule

protease with a high degree of homology to NE, does not degrade *Shigella* virulence proteins.

By specifically cleaving the *Shigella* virulence factors, NE possibly inhibits the interaction of *Shigella* with host proteins, thus preventing the escape of *Shigella* from the phagolysosome of neutrophils.

1.4 Serine Proteases: NE and CG

1.4.1 Serine Proteases

Despite their opposing specificity towards *Shigella* virulence factors NE and CG are neutral serine proteases of the same subfamily. The super-family of serine proteases contains peptidases with very diverse functions such as digestion, degradation, blood clotting, cellular and humoral immunity, fibrinolysis, fertilization, embryonic development, protein processing and tissue remodelling (Rawlings and Barrett 1993). The common feature of serine proteases is the occurrence of a highly reactive serine residue in their catalytic center. Apart from that, serine proteases are very diverse and divided into evolutionary unrelated clans (Barrett and Rawlings 1995). The clans differ in their overall structure and the succession of the catalytic residues in their primary sequences (Krem and Di Cera 2001). Clan members are subdivided into families based on sequence homology (Rawlings and Barrett 1993; Barrett and Rawlings 1995). NE and CG are members of one subfamily of the chymotrypsin-like clan (Lesk and Fordham 1996).

1.4.2 Chymotrypsin-like serine proteases

Chymotrypsin-like serine proteases combine a large group of diverse proteases, including chymotrypsin, trypsin, NE, and CG. The common features among chymotrypsin-like serine proteases are the strictly conserved geometry in their catalytic triad and their overall structure. The identical fold is composed of two asymmetric β-barrel domains and a C-terminal α-helix (figure 1.3). Enzyme-substrate interactions involve both β-barrel domains. Each barrel consists of six antiparallel β-sheets. In some family members, e.g. NE, the barrels are connected by an extra α-helix. The barrels form a cleft in which the catalytic triad and the substrate-binding sites are located (Perona and Craik 1997).

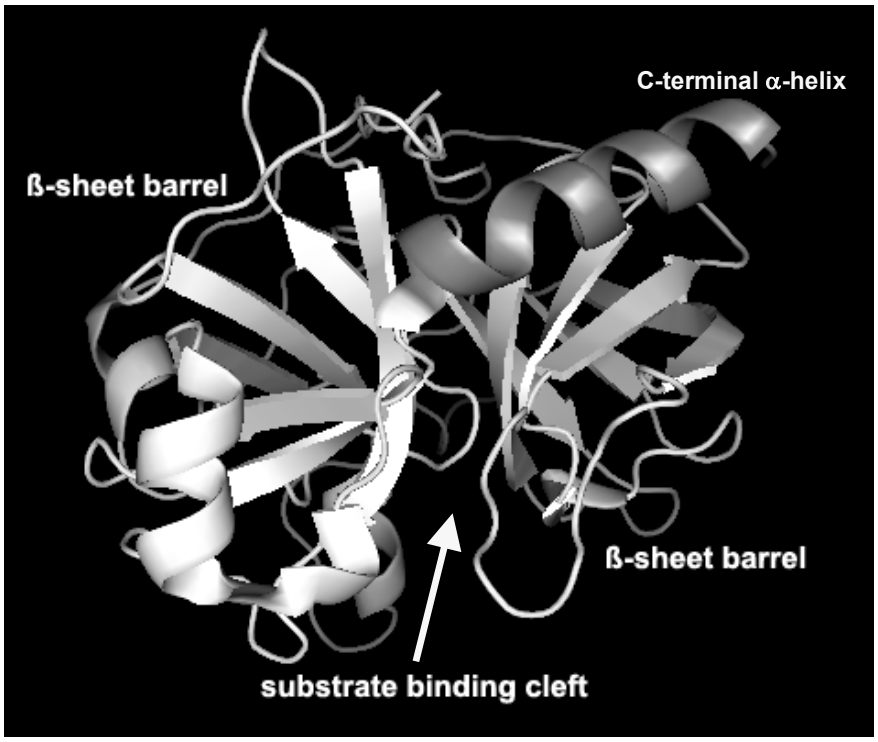


Fig. 1.3: Structure of bovine chymotrypsin.
Bovine chymotrypsin is composed of two β-barrel domains and a C-terminal α-helix. Its structure is a classical example of the common fold of chymotrypsin-like serine proteases. The catalytic triad and the substrate-binding sites are located within the cleft formed by the two β-barrel domains, which consist of six antiparallel ß-sheets each (flat arrows). The residues is shown as a cartoon. The respresentation was generated using PYMOL (DeLano 2002) and is based on the crystallization of chymotrypsin by (Pjura et al. 2000).

Catalytic triads cleave a peptide bond between the carbonyl group of the N-terminal amino acid and the amide group of the C-terminal amino acid. Residues adjacent to the scissile bond are termed P1 and P1' respectively (figure 1.4). The catalytic triad consists of serine, histidine and aspartate: Ser^{195}, His^{57}, and Asp^{102} according to the chymotrypsinogen numbering of (Hartley B.S. 1971). The serine side chain forms a hydrogen bond with the imidazole ring of histidine and this histidine shares a hydrogen with aspartate. The resulting geometric arrangement allows the three amino acids to act together in a nucleophilic attack and to cleave the peptide bond of the substrate (see also figure 1.6). The cleavage of the substrate is a coordinated multi-step process. Upon binding of the substrate, the histidine ring positions the serine side chain and polarizes the hydroxyl group of this side chain by transiently binding its hydrogen. The serine hydroxyl group is now more nucleophilic and

can attack the carbonyl group of the scissile bond. Aspartate supports the orientation of the histidine side chain and improves its proton acceptor qualities by electrostatic interactions.

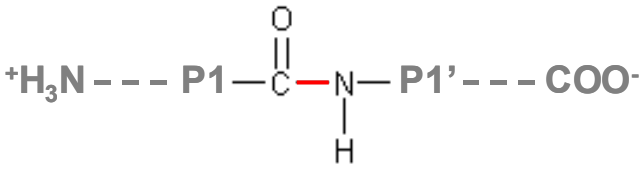


Fig. 1.4: Nomenclature of substrate residues at the scissile bond.
The scissile peptide bond is shown in red. The residues N-terminal of the bond are termed P1-Pn, whereas C-terminal residues are called P1'-Pn' (Berg JM 2003)

The nucleophilic attack leads to conformational change in the vicinity of the carbonyl carbon atom of the substrate. The previous trigonal planar structure changes to a tetrahedral one. This instable formation is stabilized by the amides of serine 195 and glycine 193. The resulting formation is called the oxyanion hole or oxyanion pocket. The peptide bond is cleaved and the carbonyl group of P1 is transiently attached to the serine side chain, whereas the amide group of P1' is bound to the histidine imidazole ring. The histidine transfers the proton of the serine to the amide group of P1' and the former C-terminal part of the substrate can dissociate from the enzyme. To release the N-terminal part of the substrate from the serine side chain, a water molecule is needed and the same steps are repeated as described before. The histidine polarizes the water molecule, which attacks the carbonyl group of P1. The carbonyl group binds the hydroxl group of the water and the P1-serine ester bond is cleaved. The N-terminal substrate dissociates, the histidine-serine hydrogen bond is reestablished and the enzyme is prepared for a new catalytic cycle (Berg JM 2003).

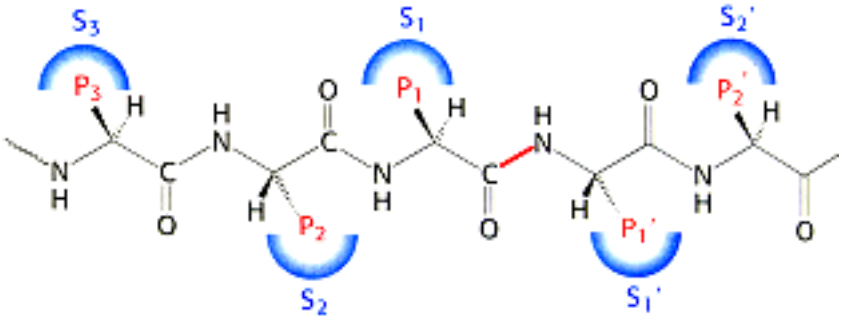


Fig. 1.5: Representation of protease-substrate interactions.
Multiple enzymatic binding sites/pockets (in blue) directly contact the P sites (in pink) of the substrate. The nomenclature of the S sites (Sn ..., S2, S1; S1', S2', ... Sn') is concordant with the P sites [(Schechter and Berger 1968); see also figure 1.4]. The scissile bond is shown in red [adapted from (Berg JM 2003)].

An important step for the proper function of the catalytic triad is the correct binding and positioning of the substrate. The substrate binding sites (S) of the enzyme play a key role in this process. These sites are composed of different amino acids and form structural pockets, which interact with the amino acid side chains of the substrate (figure 1.5).

The S1 pocket is thought to have an important role in the substrate specificity of the individual chymotrypsin-like serine proteases (Steitz and Shulman 1982). In general, the S1 pocket consists of three β-sheets from the C-terminal β-barrel domain [residues 189-193, 214-216, and 226-228 chymotrypsinogen numbering (Hartley B.S. 1971)]. The serine at position 214 is highly conserved among the chymotrypsin-like serine proteases and contributes to the S1 binding (Perona and Craik 1995). It aids in creating a polar environment for the catalytic aspartate 102 (McGrath et al. 1992). In most of these proteases, including NE and CG, the β-sheets are connected by two surface loops and the disulfide bond Cys^{191}-Cys^{220}(Perona and Craik 1997).

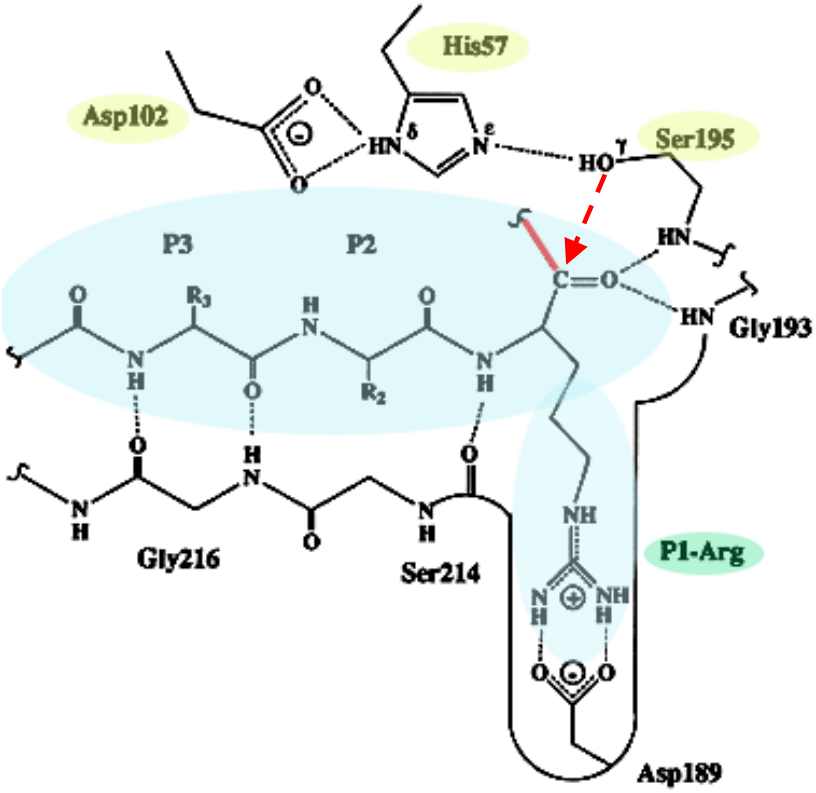


Fig. 1.6: Specificity pocket of trypsin.
A schematic representation of trypsin interacting with a peptide substrate is shown. The catalytic residues (His57, Asp 102 and Ser195, yellow) and the enzyme residues that contact substrate residues are shown (blue). The positively charged arginine side chain at position P1 of the substrate is attracted by the negatively charged aspartate 189 located at the bottom of the S1 specificity pocket. This interaction as well as five enzyme-substrate hydrogen bonds at positions P1 and P3 and glycine 193 help to position the scissile peptide bond (red) for the nucleophilic attack by the polarized hydroxyl group of Ser 195 (red arrow). The respresentation is adapted from (Perona and Craik 1997).

Besides common characteristics, specific residues differ in the S1 pocket of the individual proteases. It is assumed that these varying amino acid compositions account for the different substrate specificities (Krem et al. 1999). Trypsin, for example, prefers to cleave after an arginine or lysine at the P1 position of the substrate. This is because a negatively charged aspartate (Asp 189) is located at the bottom of the trypsin S1 pocket. The aspartate can interact with the long and positively charged side chains of arginine and lysine (figure 1.6). In contrast to that, chymotrypsin contains an uncharged serine at the bottom of its S1 pocket. Thus, uncharged, aromatic side chains of phenylalanine, tyrosine and tryptophan fit into this hydrophobic pocket. The S1 pocket of CG, for example, shows similarity to the chymotrypsin pocket (Harper et al. 1984).

Apart from the residue at the bottom of S1, other amino acids define the characteristics of the pocket through their side chains. In enzymes with trypsin or chymotrypsin specificities, amino acid 216 is a glycine (figure 1.7). Since the glycine side chain consists only of hydrogen, it allows large substrate side chains to the base of the pocket. On the contrary, in elastase-like enzymes the side chains of the amino acids at position 190 and 216 exclude large and bulky side chains to enter the S1 pocket (figure 1.7). This structural observation is in accordance with the preference of NE for valine as P1residue in peptide substrates (Harper et al. 1984), since valine only has a small alkyl side chain.

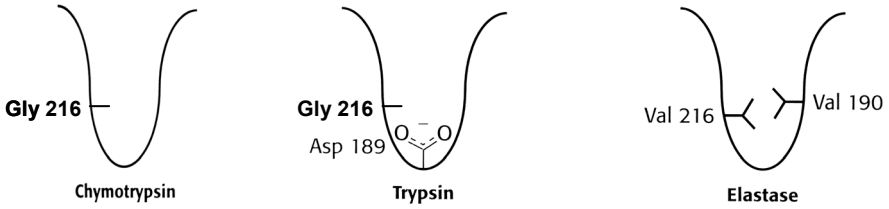


Fig. 1.7: Schematic model of S1 pockets from different chymotrypsin-like serine proteases.
The S1 pocket of chymotrypsin allows large, hydrophobic side chains to enter the pocket completely, whereas trypsin prefers long, negatively side chains. Val190 and 216 confine the S1 pocket of elastase to small alkyl side chains [adapted from (Berg JM 2003)].

Yet, the model that substrate specificities are only determined by the S1 pockets is incomplete. Substitution of individual amino acids in the S1 site of trypsin with their counterparts in chymotrypsin fails to transfer chymotryptic specificity to the mutant enzyme (Graf et al. 1987; Hedstrom et al. 1992). Transfer of specificity requires the additional exchange of amino acids in at least two distal segments of the enzyme, none of which

directly contacts the substrate (Hedstrom et al. 1992). It is notable that studies on the different S1 sites of the chymotrypsin-like serine proteases have been carried out with peptide but not with full-length protein substrates. The recognition of these protein substrates likely involves other substrate-enzyme interactions outside of the binding pockets (Perona and Craik 1995).

1.4.3 Human neutrophil cathepsin G (CG) and neutrophil elastase (NE)

Human neutrophil cathepsin G (CG; EC 3.4.21.20) and human neutrophil elastase (NE; EC 3.4.21.37) are major constituents of the antimicrobial proteins of neutrophils. At the sites of infection they are mainly released into the phagocytic vacuole and to a lesser extend to the extracellular space to exert their specific an unspecific functions. One of the specific functions of NE is the degradation of virulence factor of *Shigella* and other Gram-negative enteropathogenic bacteria. It is intriguing that CG does not cleave these effectors since the two proteins share many characteristics.

As mentioned above, CG and NE belong to the same subfamily of chymotrypsin-like serine proteases. Thus they are homologous and their primary amino acid sequence is to 37% identical. The residues of the catalytic triad are the same positions (His^{57}, Asp^{102} and Ser^{195}) and their overall fold consists of two ß-barrels. In addition to that, NE and CG show a striking structural similarity beyond their overall fold (figure 1.8). When superimposed, the α-carbons of NE and CG only display a root mean square deviation (RMSD) of 0,9 Å. The RMSD describes the difference in localization of the α-atoms at similar positions in the structure. A RSMD of zero means that the structures are identical in conformation (Maiorov and Crippen 1994). However, the crystal structures show that the S1 sites of NE and CG are different (Bode et al. 1989; Navia et al. 1989; Hof et al. 1996). The S1 pocket of CG can harbor large and bulky side chains whereas only amino acids with small alkyl side chains fit into the S1 pocket of NE. Using peptide substrates, it was confirmed that CG and NE prefer different amino acids at the P1 position: phenylalanine and lysine for CG versus valine and leucine for NE (Harper et al. 1984; Tanaka et al. 1985). In general, NE hydrolyzes peptide substrates much faster than CG (Harper et al. 1984; Tanaka et al. 1985).

Human NE and CG are processed and stored in the same manner. They are both synthesized as inactive zymogens in the premyeloid and myeloid stage of neutrophil and monocyte differentiation in the bone marrow (Campbell et al. 1989; Fouret et al. 1989). Protein synthesis seems restricted to the differentiating neutrophil, since no transcription, at least of

the NE gene (Fouret et al. 1989), is observed in neutrophils after they have left the bone marrow. Both enzymes are N-glycosylated. NE has two asparagine-linked carbohydrate chains (asparagine 95 and 144, chymotrypsin numbering), whereas CG has one potential glycosylation site [asparagine 60 (Salvesen et al. 1987; Sinha et al. 1987)]. The composition of the complex mannose oligosaccharides sugars can differ resulting in different NE and CG isoforms (Lindmark et al. 1990; Watorek et al. 1993; Kim and Kang 2000). How the glycosylated precursors are transferred to the maturing granule compartment is unclear, but obviously it is independent of the mannose-6-phophate receptor (Rijnboutt et al. 1991a; Rijnboutt et al. 1991b; Hasilik 1992; Glickman and Kornfeld 1993) which is often involved in targeting proteins to lysosomes (Kornfeld and Mellman 1989).

In the developing granule, NE and CG are post-translationally processed to mature, active proteins. The N-terminal processing of the preproenzymes involves two cleavage steps: first a signal peptide, then the two-residue activation peptide is cleaved off (Salvesen and Enghild 1990; Brown et al. 1993; McGuire et al. 1993; Urata et al. 1993). Both mature proteins start with an isoleucine. This N-terminal processing seems essential for activity of the protein, since recombinant expression of full-length NE or mature NE starting with a methionine did not yield active proteins (Li and Horwitz 2001). In addition, the C-terminus of both enzymes is processed to its individual mature form (Salvesen and Enghild 1990; Gullberg et al. 1994). However, the carboxyl prodomains do not seem to be important for proper targeting and enzymatic activity (Gullberg et al. 1995). Finally, the 267 amino acid NE precursor is processed to its 218 amino acid mature form and the CG 255 amino acid precursor results in a 226 amino acid mature CG protein.

The active proteins are predominantly stored in the azurophilic granules of neutrophils in fairly high concentrations [1-2 pg/cell, (Wiedow et al. 1996)]. NE and CG are very basic with a PI of > 9 and ~12, respectively, and through their arginine residues they are probably anchored to the negatively charged heparin and chondroitin sulfate proteoglycan matrix of the granule. Upon activation, neutrophils discharge their granule contents into the bacteria containing vacuole or to the extracellular space. A minimal proportion of the active proteins is also localized on the surface of unstimulated neutrophils (Owen et al. 1995). This surface expression is increased when the neutrophils are exposed to chemoattractants such as fMLP (Owen et al. 1995). The correct distribution of NE in the granules and on the surface seems important to prevent neutrophil deficiencies (neutropenia). Hereditary neutropenia is rare but predisposes people to infections. In cyclic neutropenia (CN) the neutrophil number in the blood oscillates from zero to normal (Morley et al. 1967; Lange 1983) whereas in severe

congenital neutropenia (SCN) the total neutrophil number is drastically reduced (Ancliff et al. 2001). All cases of CN and 75% of SCN cases are caused by mutations in the NE gene (Horwitz et al. 1999). These mutations are thought to interfere with the correct targeting of NE from the trans-golgi network to the granules and the plasma membrane (Horwitz et al. 2004).

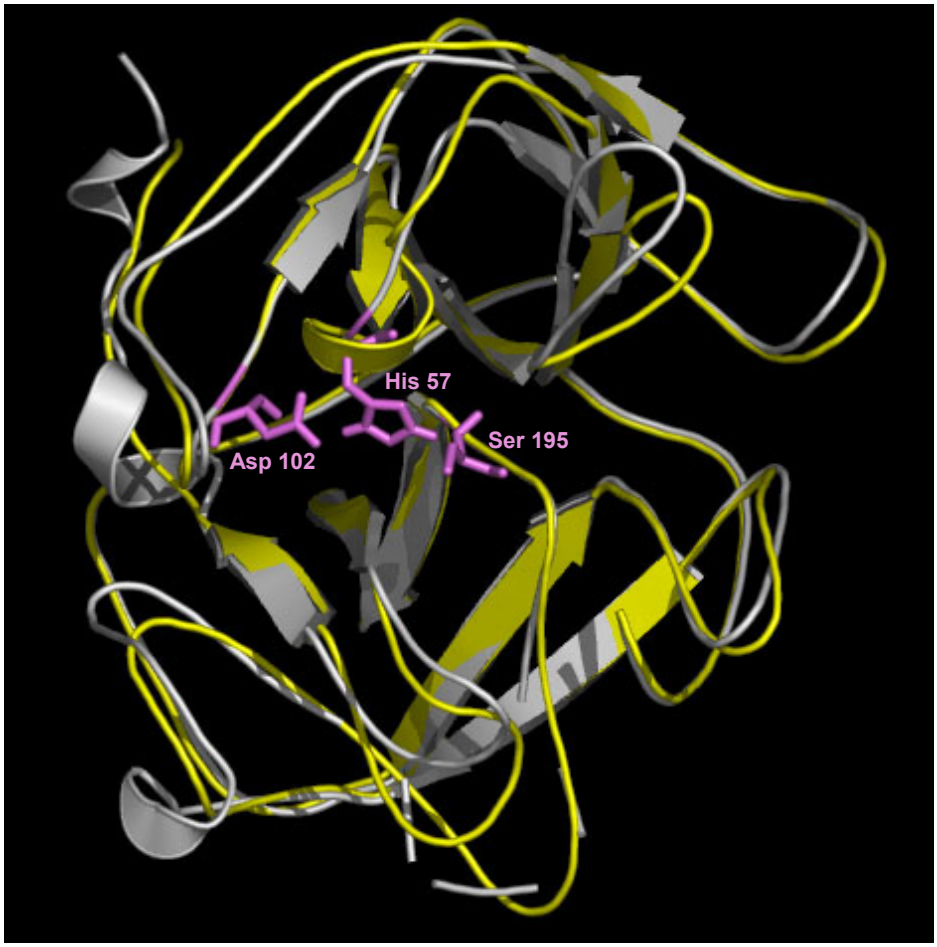


Fig. 1.8: Superimposition of the NE and CG crystal structures.
190 α-carbons of the crystal structures of NE (yellow) and CG (grey) were superimposed with a root mean square deviation (rmsd) of 0,9 Å. The catalytic triad consisting of histidine (position 57), aspartate (102) and serine (195) is shown in purple. The superimposition was achieved using the SWISS-PDB-VIEWER (Guex 1997).

Despite its low activity towards peptide substrates, CG, like NE, unspecifically degrades extracellular matrix components such as proteoglycan, collagen, laminin, fibronectin and even elastin (Roughley 1977; Roughley and Barrett 1977; Baggiolini et al. 1979; Caughey 1994). These proteolytic characteristics may be helpful for the neutrophil egress from the

bloodstream and their subsequent transmigration through tissue. But the role of NE and CG in these processes remains controversial (Shapiro 2002).

At sites of inflammation, neutrophils degranulate and discharge NE and CG mainly into the phagocytic vacuole and to a lesser extent into the extracellular space (Faurschou and Borregaard 2003). When exposed to the extracellular environment, the enzymes are either membrane-bound, part of neutrophil extracellular traps (NETs) (Brinkmann et al. 2004), or freely released. To avoid digestion of healthy tissue NE and CG levels are tightly controlled by their physiological inhibitors, α1-antitrypsin and α1-antichymotrypsin, respectively (Beatty et al. 1980). These plasma-derived inhibitors belong to the family of serpins (serine protease inhibitors) and are highly abundant extracellular proteins (Shapiro 2002). They can inhibit free but not membrane-bound NE and CG (Owen et al. 1995). Hereditary deficiency in α-antitrypsin causes chronic destruction of alveolar walls and leads to pulmonary emphysema development (Gadek et al. 1981; Eriksson 1984). Deficiencies in α1-antichymotrypsin predispose patients for lung diseases (Faber et al. 1993). In addition, NE is present in cystic fibrosis airways (Delacourt et al. 2002) and induces excess mucus production in chronic obstructive pulmonary disease (COPD) (Shapiro 2002).

Apart from their degradative properties, NE and CG are thought to be important regulatory tools in inflammatory processes (Wiedow and Meyer-Hoffert 2005). For example, NE induces IL-8 expression via toll-like receptor 4 *in vitro* (Devaney et al. 2003) and CG activates PAR-4 (protease-activated receptor) to initiate thrombocyte aggregation (Sambrano et al. 2000).

The importance of NE and CG in immunity is proven by various studies. Mice deficient in NE and / or CG are susceptible to fungal infections despite normal neutrophil development and recruitment (Tkalcevic et al. 2000). The same study indicates that NE and CG act as effectors in the endotoxic shock cascade downstream of TNFα. In accordance with the observed specificity of NE for virulence factors of Gram-negative bacteria, mice deficient in NE have impaired survival following infections with gram-negative pathogens. In contrast, mice deficient in CG are more susceptible to Gram-positive bacteria (Belaaouaj et al. 1998; Reeves et al. 2002). Interestingly, CG shows an antimicrobial activity that is independent of its enzymatic function (Bangalore et al. 1990).

1.5 Aim of study

As described above, NE is a key component in the resolution of a *Shigella* infection by neutrophils. NE specifically targets *Shigella* virulence factors but not proteins that are not associated with virulence or that are important for bacterial homeostasis (Weinrauch et al. 2002). Therefore, NE is likely to modify the interaction of *Shigella* with host cells, as seen in macrophages or epithelial cells upon *Shigella* uptake. Indeed, in neutrophils with pharmacologically inhibited or genetically inactive NE, *Shigella* can escape into the cytoplasm and its survival rate is increased (Weinrauch et al. 2002). The specificity of NE for virulence factors holds also for other Gram-negative pathogens such as *Salmonella* and *Yersinia* (Weinrauch et al. 2002). Furthermore, CG, another abundant granule protease, does not degrade *Shigella* virulence proteins, although it is homologous to NE and their crystal structures are almost identical. Therefore we raised the question why NE but not CG targets virulence factors and how NE distinguishes virulence factors from other bacterial proteins. The aim of this study was to understand how NE recognizes virulence factors. To this end, we first analyzed the substrate for a NE recognition motif in the primary or higher order structures. As example we used the *Shigella* virulence factor IpaB. Secondly, we addressed the question of the NE specificity by a functional analysis of NE mutants. These mutants were generated on the basis of a structural comparison of NE and CG and were tested for their ability to cleave the *Shigella* virulence factors IpaB and IcsA.

2 Materials and Methods

2.1 Bacteria – *Escherichia coli*

2.1.1 Strains

E. coli TOP 10: F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 *lac*X74 *rec*A1 *ara*D139 (*ara- leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G (Invitrogen, chemically competent)

E.coli Rosetta™(DE3) pLys:
F$^-$ *omp*T *hsd*S($r_B^-m_B^-$) *gal dcm* Tet^r *lacY1* (DE3) pLys SRARE (Cam^r) (Novagen, chemically competent)

DE3 indicates that the *E.coli* Rosetta™(DE3) pLys strain is a lysogen of lambda DE3, and therefore carries a chromosomal copy of the T7 RNA polymerase gene. This gene is under control of the lacUV5 promoter, which is inducible by addition of IPTG.
The DNA encoding for the recombinant protein was under control of the T7 promoter. To avoid basal expression of the polymerase and thus unspecific expression of the cDNA encoding for the recombinant protein, the bacteria contained the pLys plasmid. This plasmid encoded for T7 lysoszyme, a natural inhibitor of T7 RNA polymerase (Studier 1991; Zhang and Studier 1997). To enhance the expression of eukaryotic proteins, the bacteria additionally contained tRNAs for seven codons rarely used in *E. coli* and other prokaryotes (AGA, AGG, AUA, CUA, GGA, CCC, and CGG). The tRNA genes were also encoded on the pLys plasmid that additionally carried a chloramphenicol resistance marker. This plasmid was compatible with the plasmid pET-28(a)+ which was used to express NE.

2.1.2 Growth conditions and media

E.coli strains used for plasmid amplification were cultured according to standard procedures (Sambrook and Russell 2001) at 37°C in Luria-Bertani (LB) medium supplemented with either ampicillin (100 μg/ml) or kanamycin (50 μg/ml). *E.coli* Rosetta™(DE3) pLys used for protein expression was grown in LB medium supplemented with chloramphenicol (50 μg/ml). If the bacteria carried the expression plasmid pET-28a(+), the media was

additionally supplemented with kanamycin (50 µg/ml). Subcultures were diluted 1:100 in LB medium without antibiotics.

2.1.3 Protein expression

E.coli Rosetta™(DE3) pLys were transformed with the empty expression plasmid (pET-28a(+)) or with the plasmid carrying the respective cDNA encoding for histidine tagged mature NE (pET-28a(+)/NE mature). Transformation was carried out according to the manufactures protocol. Transformed bacteria were plated on LB agar plates supplemented with chloramphenicol (50 µg/ml) and kanamycin (50 µg/ml). Overnight cultures were grown in LB medium with kanamycin (50 µg/ml) and subcultured at a 1:100 dilution. Protein expression was induced by addition of IPTG (1mM) to the subculture at an OD_{600} of 0,8 for 4h. To test for expression of the recombinant proteins, 1 ml aliquots of the subcultures were harvested and dissolved in Laemmli buffer (Laemmli 1970). The samples were separated via SDS-PAGE and analyzed by immunoblotting or Coomassie staining.

2.1.4 Protein purification

Protein purification was carried out at 4°C or on ice. 2 l subculture of *E.coli* Rosetta™(DE3) pLys carrying pET-28a(+)/NE mature were harvested. The pellet was dissolved in 20 ml lysis buffer (150 mM NaCl, 50 mMTris-Cl, pH 8,0, 5 mM DTT, 10 mm Imidazole, 0,2 % v/v triton 20 µg/g E.coli DNAse) using sonication (Sonoplus 2070, Bandelin Corp, 5x 45sec, 40% power, level 5). After centrifugation (3 h, 22000 x g) the supernatant was applied to a nickel column (Ni-NTA Agarose, Qiagen) that had been equilibrated (10 mM imidazole, 150 mM NaCl, 25 mM sodium phosphate buffer, pH 8). The flowthrough was reapplied to the column. Following two washing steps (2 x 10 ml; 50 mM imidazole, 150 mM NaCl, 25 mM sodium phosphate buffer, pH 8), the proteins were eluted using a high imidazole concentration (250 mM imidazole, 150 mM NaCl, 25 mM sodium phosphate buffer, pH 8). At every purification step aliquots were taken and analyzed by SDS-PAGE and subsequent immunoblotting or Coomassie staining. The fractions of the eluate containing the histidine tagged recombinant NE protein were combined and dialysed for 14 h. The dialysis buffer was changed twice during that time (36 mM Na-acetate, 164 mM glacial acid, pH 4). The dialyzed eluate was subsequently lyophilized and dissolved in 500 µl pNE storage buffer (20 mM Na Acetat/150 mM NaCl, pH 4,0).

2.2 Bacteria – *Shigella flexneri*

2.2.1 Strains

M90T:	wildtype strain of *S.flexneri,* serotype 5a
M09T ΔipaB + pUC19:	(Guichon et al. 2001)
M90T ΔipaB pUC19/wt IpaB:	(Guichon et al. 2001)
*M90T*ΔipaB pUC19/Δ 8-10 aa IpaB:	(Guichon et al. 2001)

2.2.2 Growth conditions and media

Shigella was grown on TSA plates (Difco^{TM} Tryptic Soy Agar, BD) including 0.01% Congo red. *Shigella's* ability to bind Congo red correlates with the presence of the virulence plasmid (Qadri et al. 1988). For overnight cultures, a single colony from a plate was grown in 5 ml TSB-medium (Bacto^{TM} Tryptic Soy Broth, BD) at 37°C shaking at 200 rpm. *S. flexneri* carrying the pUC19 plasmid was cultured in TSB medium supplemented with 100 μg/ml ampillicin. Overnight cultures were subcultured 1:100 in TSB without antibiotics. An OD_{600} of 0.1 corresponds to a concentration of 4 x 10^7 bacteria/ml.

2.2.3 Cleavage of IpaB

Subcultures of *M90T* ΔipaB pUC19/wt ipaB and *M90T*ΔipaB pUC19/Δ 8-10 aa ipaB were grown for 4-5 h at 37°C, shaking at 200 rpm. The bacteria were pelleted for 15 min at 4°C, 17600 x g. The supernatant was filtered (0,22 μm pore size) on ice and mixed with sodium phosphate buffer (20 mM, ph 7,5). For the experiments shown in figure 3.2 and 3.7 the supernatant was used immediately. For all other IpaB cleavage experiments, supernatant of *M90T* ΔipaB pUC19/wt ipaB was generated once and stored in 1 ml aliqots at –20°C. For the experiments aliquots were thawed and mixed with pNE or the individual cell lysates. To denature IpaB, thawed supernatant was heated for 10 min at 95°C and immediately cooled on ice prior to addition of pNE or the lysates. All cell lysates had been generated from 1x10^7 cells. The lysates had been incubated with the inhibitor cocktail for 15 min at RT prior to addition to the supernatant except for the experiments shown in figure 3.2 and 3.7. pNE and cell lysates were added to supernatant and incubated as indicated in the individual figure legends. Proteins were TCA precipitated and dissolved in Laemmli buffer (Laemmli 1970).

$1x10^8$ bacterial equivalence were analyzed with SDS-PAGE at a polyacrylamide concentration of 12% and subsequent immunoblotting using an IpaB antibody.

2.2.4 Cleavage of IcsA and OmpA

M90T were grown overnight and subcultured for 2h. The concentration of the bacteria was determined at an OD of 600 nm wave-length. The bacteria were harvested for 10 min at 4°C, 5500 x g and the supernatant was discarded. The M90T were dissolved in 1x PBS (Gibco) at concentration of $3x10^9$/ml and kept on ice during this procedure. $3x10^8$ bacteria in 100 µl PBS were mixed with 900 µl IcsA-cleavage buffer (4,5 g NaCl, 4 g nutrient broth (BD) in 500 ml; 20 mM sodium phosphate, pH 7,4) and incubated with pNE and the individual cell lysates. All cell lysates had been generated from $1x10^7$ cells and the lysates had been incubated with the inhibitor cocktail for 15 min at RT prior to addition to the bacteria. pNE and lysates were added to the bacteria as indicated in the individual figure legends and incubated for 1h at 37°C, shaking at 180 rpm. The bacteria were centrifuged for 5 min at 4°C, 12000 x g and dissolved in Laemmli buffer (Laemmli 1970). For IcsA detection, $7,5x10^7$ bacterial equivalence were analyzed by a SDS-PAGE at a polyacrylamide concentration of 10% and subsequent immunoblotting using an IcsA antibody. For OmpA detection, $2x10^7$ bacterial equivalence were analyzed by SDS-PAGE at a polyacrylamide concentration of 15% and subsequent immunoblotting using an OmpA antibody.

2.3 Cells

2.3.1 Cell line

RBL-1	rat basophilic leukemia cells (DSMZ, #ACC 147)

2.3.2 Growth conditions and media

RBL-1 cells were cultured in RPMI media supplemented with 1% L-glutamine, 1% Na-pyruvate and 10% FCS (complete media) at 37°C, 5% CO_2. Stably transfected cell lines were cultured in complete media supplied with geneticin (0,5 mg/ml; Gibco). All cell culture equipment was sterile and purchased from Gibco unless stated otherwise. The RBL-1 cells grow both non-adherent (3/4) and adherent (1/4). In order to split the cells, the

adherent fraction was incubated with trypsin (0,05% w/v in PBS) and collected with the non-adherent fraction by centrifugation. The concentration of the cells was determined using a Neubauer counting chamber (0,0025 mm^2, Labor Optik). Cells were seeded at a concentration of $1x10^6$/ml.

2.3.3 Transient transfection

A total of $1x10^6$ RBL-1 cells were seeded in a 10 cm petridish in complete media and co-transfected with either 4 µg pCS2+/NE and 1 µg pCS2+/βgal or with 4 µg pCS2+ and 1 µg pCS2+/βgal. In separate tubes, DNA and Lipofectamine 2000 (Invitrogen) were diluted in 250 µl Opti-MEM1 buffer and mixed gently. After an incubation period of 5 min at RT both solutions were combined, mixed gently and incubated for another 20 min at RT before addition to the cells. The cells were cultured for 24 h in complete medium without geneticin. Then theywere harvested, washed in 1x PBS and dissolved in 150 µl 250 mM Tris-Cl, pH 7,5. As a transfection control, 30 µl of the cells were analyzed for β-galactosidase activity. The remaining 120 µl were lysed according to the protocol described below. The complete lysate was tested for NE activity.

2.3.4 Stable transfection and single-cell dilution

RBL-1 cells were separately transfected with the empty expression vector (pcDNA3), the expression vector carrying the DNA encoding for the wildtype full-length NE protein (pcDNA3/NE), or with the expression vector carrying the DNA encoding for the different mutant full-length NE proteins (see table 2.1). $1x10^6$ RBL-1 cells in a total volume of 2 ml were seeded in a single well of a 6-well plate and transfected as described above using 4 µg DNA. The following day, the media was exchanged and the cells were incubated in complete media for another 48h before addition of geneticin. Since the expression vector pcDNA3 carries a geneticin resistance marker, successfully transfected cells were viable in this media after an incubation period of 5 days. Geneticin resistant cells were separated from dead cells by Ficoll centrifugation (50 min at RT, 400 x g), and seeded into individual 96-wells at a concentration of 0,5 cells/well to obtain one cell per well. The single-cell derived clones were expanded and NE enzymatic activity was tested from several of the different clones after growth to confluence as described below.

2.3.5 Cell lysis

Cell lysates were generated according to a modified protocol described by (Li and Horwitz 2001). Cells ($1x10^{5-8}$) were pelleted and washed in sterile 1x PBS (Gibco). The pellets were either stored at –80°C or resuspended in 120 µl of 250 mM Tris-Cl, pH 7,5. The concentration was adjusted to 100 mM Tris-Cl, pH 7,5, 1 M $MgCl_2$, 0,1% Triton X-100 in a 300 µl volume followed by one freeze-thaw cycle at –20°C overnight. The cell suspension was thawed on ice and DNA was fragmented by sonication (2 x 20 sec, position 3, 20 % power; Sonoplus 2070, Bandelin). Following this, the volume of the suspension was increased to 500 µl (700 mM NaCl, 60 mM Tris-Cl, pH 7.5, 600 mM $MgCl_2$, 0,1% Triton X-100). In order to purify the lysate from cell debris, it was centrifuged at 16000 x g at 4 °C for 90 min. 450 µl supernatant was collected and adjusted to a final volume of 600 µl (100 mM Tris-Cl, pH 7,5, 1 M NaCl, 500 mM $MgCl_2$, 0,1% Triton X-100).

2.4 Chemicals

All chemicals were obtained from Sigma-Aldrich unless stated otherwise.

2.5 Enzymatic Assays

2.5.1 β-galactosidase activity

Transiently transfected cells were assayed for β-galactosidase expression using the Galacto Light-Plus Kit from Applied Biosystems. 30 µl of each sample was mixed with 250 µl lysis buffer, centrifuged for 2 min at 4°C, 13000 x g. 20 µl of supernatant were analyzed for β-gal activity using a microtiterplate luminometer (BD Biosciences) according to the manufacturers' protocol Every measurement was done in triplicates.

2.5.2 Reagents to measure NE and CG activity

pNE and pCG were both purified from human sputum (Elastin Products Company). NE activity was measured using the NE peptide substrate N-methoxy-succinyl-alanine-alanine-proline-valine-pnitroanilide (MeO-Suc-AAPV-pNA, 20,3 mM in 1-methyl-2-

pyrrolidinone). To specifically inhibit the NE protein, the inhibitor N-methoxy-succinyl-alanine-alanine-proline-valine-chloromethyl ketone (NE-CMK, 100 mM in DMSO) was utilized. In order to measure CG activity, the CG peptide substrate N-succinyl-alanine-alanine-proline-phenylalanine-p-nitroanilide (N-Suc-AAPF-pNA, 60,8 mM in 1-methyl-2-pyrrolidinone) was used. The activity of CG was specifically inhibited by the peptide benzyloxycarbonyl-glycine-leucine-phenylalanine-chloromethyl ketone (Z-GLF-CMK, 10 mM in DMSO).

To block degradation of IpaB and IcsA by endogenous RBL-1 proteases, cell lysates were mixed with an individually prepared inhibitor cocktail (IC) and incubated for 15 min at RT. The IC was composed of bestatin, chymostatin, E-64, EDTA, leupeptin, pepstatin A, and TPCK at a concentration of 1mM each. The individual inhibitors were dissolved according to the manufacturers' instructions and combined in DMSO. The concentration of the IC in the cell lysates corresponded to 50 µM, except for the experiment shown in figure 3.9 were 20 µM was used

2.5.3 NE and CG activity of cell lysates

The NE and the CG peptide substrate consist of four specific amino acids coupled to a chromophore (nitroanilide) at the P1 position. Cleavage by NE or CG results in the release of the chromophore leading to an increase of the optical density (OD) when measured at 410 nm wave-length. The complete lysate of transiently transfected cells or $1x10^5$ and $1x10^8$ cell equivalents of stably transfected cell lines (final volume of 600 µl) were mixed with 20 µl NE peptide substrate or with 20 µl CG peptide substrate. The mixtures were incubated for 30 min at 37°C in the dark. The reaction was terminated by addition of 300 µl of PMSF/PBS [1mM PMSF in 1x PBS (Gibco)]. The OD was measured at 410 nm wave-length using a photometer (Ultrospec 2100, Amersham). The lysate from cells transfected with the empty expression vector (vector lysate) was used for normalization. Lysate of cells expressing wildtype NE (wt) or vector lysate containing pNE was used as positive control for NE activity. pCG was added to vector lysate and served as positive control for CG activity.

If NE and CG specific inhibitors were used, they were added to the lysates before adding the substrates and this mixture was incubated at room temperature for 15 min. The final concentrations of the purified enzymes or the cell lysates are indicated in the individual figure legends.

2.5.4 NE and CG kinetics / activity units

The NE peptide substrate (10 µl/ml assay buffer: 500 mM NaCl, 200 mM Tris-HCl, pH 8) and the CG peptide substrate (70 µl/ml assay buffer: 100 mM Tris-HCl, ph 8,3) were dissolved in the individual assay buffers and protected from light. 750 µl of the respective substrate/assay buffer mixture were added to a plastic cuvette and normalized. PNE, pCG or cell lysates were subsequently added at a volume of 250 µl and the cleavage of the respective peptide substrate was monitored by measuring the OD at a wave-length of 410 nm over 3 min every 30 sec. The NE and the CG peptide substrates consist of four specific amino acids coupled to a chromophore (nitroanilide) at the P1 position. Cleavage by NE or CG results in the release of the chromophore leading to an increase of the optical density (OD) when measured at 410 nm wave-length.

The final concentrations of the purified enzymes or the cell lysates are indicated in the individual figure legends. If necessary, the volumes of the samples were increased to 250 µl with HBSS+/10mM HEPES buffer. Within each experiment the amount of HBSS+/10mM HEPES added was equal.

If the kinetics of the individual samples were linear, the NE or CG activity units could be calculated by subtracting the OD_{410} measured after 90 sec from the one measured after 150 sec. The resulting OD/min represented the unit of NE or CG activity.

2.6 Molecular cloning techniques

Standard molecular cloning techniques were performed according to (Sambrook and Russell 2001). Plasmids and primers used in this study are listed in Table 3.1. The individual cloning strategies for expression of NE in *E. coli* and RBL-1 cells and the protocol for mutagenesis of *ELA2* (NE gene) are described below. The sequences of all cloned NE fragments were confirmed by sequencing. All restriction enzymes used in this study were purchased from NEB Biolabs. Plasmids were amplified using chemically competent *E. coli* (TOP10, Invitrogen) according to manufacturers' guidelines. Plasmid DNA was extracted from *E. coli* by the alkaline lysis procedure (Sambrook and Russell 2001), and further purified using the Qiagen plasmid kits.

2.6.1 Cloning of NE for expression in E. coli

The DNA encoding for the human mature NE protein was amplified by PCR from the cDNA of NE (RZPD, #p998K167196). The PCR product was ligated into the expression vector pET-28a(+) using the restriction enzymes BspH I and Xho I, thereby joining the 3'-end of the NE DNA to a 6x hisitidine tag encoded in the vector. The relevant restriction sites had been introduced in the PCR primers of the NE amplicon. Furthermore, the forward primer contained five basepair exchanges (table 3.1, in bold) that did not alter the amino acid composition of the protein but disrupted continuous "GC" stretches at the 5'-end of the wildtype NE DNA. These basepairs were selected based on the software program PROTEOXPERT from Roche [www.proteoexpert.com (Roche-Applied-Science)]. The PCR reaction was carried out in a 50 µl volume containing 2.5 U Pfu Turbo polymerase (Stratagene), 5 µl of the respective 10x polymerase buffer, 125 ng of each of the two primers and 0.25 mM of each dNTP. Template DNA was added in variable concentrations (by default 100 pg DNA). The amplification reaction was as following: 5' 95°C, 30 cycles [1' 95°C, 45'' annealing at 60°C, 1' elongation at 72°C], and 10' final elongation at 72°C.

2.6.2 Cloning of NE for expression in RBL-1 cells

The DNA encoding for the human full-length NE protein was amplified by PCR from the cDNA of NE (RZPD, #p998K167196). The PCR product was ligated into the expression vector pCS2+ (Rupp et al. 1994; Turner and Weintraub 1994) using the restriction enzymes EcoR I and Xba I. The same restriction enzymes were used to subclone the NE fragment into the expression vector pcDNA3 for stable transfection of RBL-1 cells. The PCR reaction was identical to the one described above except that the annealing temperature was increased to 62°C.

2.6.3 Mutagenesis of ELA2 (NE gene)

Site-directed mutations in the DNA encoding for the human full-length NE protein were created using pcDNA3/NE as template. Mutations were introduced using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. This method uses complementary oligonucleotides encoding the desired mutation. The sense strand oligonucleotides used in the mutagenesis reactions are listed in table 3.1 and the introduced basepairs are highlighted in bold. All mutations were confirmed by sequencing.

Table 3.1: Plasmids and Primers used in this study.

	Primers	**Source[a]**
pCMV-Sport6/human NE cDNA		RZPD
pET-28a(+)		Novagen
pET-28a(+)/ NE mature	FW[b]: 5' –cgtcggtcatgattgt**a**gg**t**gg**t**cg**t**cg**t**gcgcggccccacgc– 3' RV[c]: 5' -ccgctcgagttggatgatagagtcg– 3'	
pCS2+		R. Rupp
pCS2+/βgal		R. Rupp
pCS2+/NE	FW[b]: 5' -gacttcaggaattcgccaccatgaccctcggccgccgactcg- 3' RV[c]: 5' -gacttcagtctagatcagtgggtcctgctggccgggtccgg-3'	
pcDNA3		Invitrogen
pcDNA3/NE		
pcDNA.3/NE 35-41	5' -gcccttcatggtgtccctgcag**atccagagcccagcaggtcagagcag a**tgcggcgccaccctgattgcgc- 3'	
pcDNA.3/NE 58A-6l	5' –cgtcatgtcggccgcgcactgc**tggggaagcaatataaatgtc**gcggtg cgggtggtcctgggagcc- 3'	
pcDNA.3/NE N98A	5' –cggctacgaccccgta**gct**ttgctcaacgacatcg- 3'	
pcDNA.3/NE N98L	5' –cggctacgaccccgta**ctg**ttgctcaacgacatcg- 3'	
pcDNA.3/NE F192A	5' –gaggggccggcaggccggcgtctgt**gcc**ggggactccggcagccccttg gtctgc- 3'	
pcDNA.3/NE F192K	5' –gaggggccggcaggccggcgtctgt**aaa**ggggactccggcagccccttg gtctgc- 3'	
pcDNA.3/NE A213V	5' –gctaatccacggaatt**gtg**tccttcgtccggggag- 3'	
pcDNA.3/NE F215A	5' –gctaatccacggaattgcctcc**gcc**gtccggggaggctgcgcctcag- 3'	
pcDNA.3/NE F215Y	5' –cgtcatgtcggccgcgcactgc**tggggaagcaatataaatgtc**gcggtg cgggtggtcctgggagcc- 3'	
pcDNA.3/NE 216-218	5' –ctaatccacggaattgcctccttc**ggcaaaagctcc**tgcgcctcagggctc taccccg- 3'	
pcDNA.3/NE 216-224	5' –ctaatccacggaattgcctccttc**ggaaagtcgtcaggggttcct**cccga tgcctttgccccggtg- 3'	

[a]Unless indicated otherwise, plasmids were constructed during the course of this study.
[b]FW = forward primer
[c]RV = reverse primer

2.7 Protein analysis

2.7.1 Determination of protein concentrations

The total protein concentration of the cell lysates and of pNE as well as pCG was determined by Bradford analysis (Bradford 1976) using the BioRad protein assay reagent and bovine serum albumin (Promega) as protein standard.

2.7.3 TCA precipitation

10% trichloroacetic acid (TCA; Merck) was added to the bacterial supernatants and incubated on ice for 30 min. The samples were centrifuged at 15000 x g for 30 min and the

pellet dissolved in acetone to reduce TCA contamination. After a 10 min centrifugation step at 15000 x g the acetone was removed and the sample left to dry (Rehm 2002). The precipitated proteins were dissolved in Laemmli buffer (Laemmli 1970).

2.7.4 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Denaturing gel electrophoresis can resolve complex protein mixtures into hundreds of bands on a gel. In SDS polyacrylamide gel electrophoresis (SDS-PAGE) separations, migration of the proteins is determined not by intrinsic electric charge of polypeptides but by molecular weight. Sodium dodecyl sulphate (SDS) is an anionic detergent that denatures proteins by wrapping the hydrophobic tail around the polypeptide backbone and thus confers a net negative charge to the polypeptide in proportion to its length. Because molecular weight is essentially a linear function of the peptide chain length, the proteins separate by molecular weight (Rehm 2002).

SDS-PAGE was always performed with the vertical Mini-PROTEAN® 3 system from BioRad. Samples were dissolved in Laemmli buffer (Laemmli 1970) and applied onto precast polyacrylamide gels (Biorad) with varying polyacrylamide concentrations. The NE samples from the bacterial expression of NE were run on 15% acrylamide SDS gels. The IpaB, IcsA and OmpA samples were run on 12, 15, and 10% acrylamide SDS gels respectively. The acrylamide gels were completely covered with running buffer (25 mM Tris, pH 8.6, 192 mM glycine and 0.1% SDS) and the proteins of the samples were separated for 1,5 h at 150 V. The separated proteins were either visualized by immunoblotting or by Coomassie staining.

2.7.5 Immunoblotting

Sufficiently separated proteins in an SDS-PAGE can be transferred via an electric current to a solid membrane for immunoblot analysis, also called Western Blot analysis. Transfer to nitrocellulose membranes (Amersham Pharmacia) was accomplished by blotting with the BIO-RAD Tank Transfer System for 1 hour at 100 V in transfer buffer (39 mM glycine, 48 mM Tris, 0.037% SDS, 20% methanol). The membranes were blocked with 3% (w/v) bovine serum albumine (BSA) dissolved in PBS (Gibco) containing 0.1% (v/v) Tween for 1 hour at RT or overnight at 4^{o}C. The primary antibodies, dissolved in the blocking buffer supplemented with 0,02% (v/v) Na-azide, were incubated with the membranes for 1-2 h at RT. The primary antibody was washed off in PBS with 0,1% (v/v) Tween three times for 10

min. The membrane was then incubated with the appropriate secondary antibody, which was dissolved in the blocking buffer, for 30-60 min at RT. The secondary antibody was also washed off in PBS with 0,1% (v/v) Tween three times for 10 min. All secondary antibodies (Jackson Laboratories Inc) were horseradish-peroxide (HRP) conjugated and bands were visualized by the ECL Western blotting detection reagents (Amersham Pharmacia). Horseradish peroxidase catalyses the oxidation of luminol, which in its excited state emits light (chemiluminescence). Enhanced chemiluminescence is achieved by the addition of chemical enhancers such as phenols, which increases the light output approximately 1000 fold. Primary and secondary antibodies used are listed in table 3.2.

Table 3.2: Primary and secondary antibodies used in this study.

Primary Antibodies	**Dilution**	**Source**
anti-pentahistidine, monoclonal	1:2000	Qiagen
anti-IpaB; monoclonal	1:5000	P.J. Sansonetti
anti-IcsA; polyclonal raised in goat	1:1000	P.J. Sansonetti
anti-OmpA; polyclonal raised in goat	1:1000	K. S. Kim
Secondary Antibodies		
anti-mouse	1:5000	Jackson Laboratories Inc
anti-rabbit	1:5000	Jackson Laboratories Inc

2.7.6 Coomassie staining

After electrophoresis the acrylamide gels were incubated with the Coomassie Blue solution (0.1% w/v Coomassie Brilliant Blue R-250 [BioRad], 20% MeOH, 10% acetic acid) at RT between 2 and 16 h to visualize proteins. Gels were destained in 50% (v/v) MeOH with 10% (v/v) acetic acid until protein bands were clearly visible.

2.8 Structure analysis

The structures of NE and CG were analyzed using PYMOL (DeLano 2002). Unless stated otherwise, the structures were presented as cartoons showing the backbones of the structures. Secondary structures like β-sheets and α-helices are depicted as flat arrows and helices respectively. Individual amino acids were highlighted and shown as sticks. The superimposition of the NE and CG structures was achieved using Swiss-PdbViewer (Guex 1997). 190 α-carbonyl atoms of the proteins were superimposed with a root mean square deviation (RMSD) of 0,9 Å. In detail, the following segments were aligned: I16-Q34, R41-W59, S61, N65-H91, Q93-G151, D153-R164, G167-C168, Q180-G184, E187-K217 S219-G220, and P225-M242. The nomcenclature and numbering is based on cathepsin G. The RMSD describes the difference in localization of the α-atoms at similar positions in the two proteins. Therefore the RMSD measures the similarity of the three dimensional structures. A RSMD of zero means that the structures are identical in conformation (Maiorov and Crippen 1994).

3 Results

The substrate specificity of NE is poorly understood. Studies using synthetic peptides have shown that NE prefers valine at the P1 position in its substrates (Harper et al. 1984). However, this preference cannot explain why NE cleaves virulence factors but not other proteins of *Shigella*, because the number of valines in the proteins of both groups is comparable. Moreover, incubation of *Shigella* virulence factors with purified NE (pNE) resulted in discrete cleavage products. This suggests that the *Shigella* proteins were folded in the *Shigella* culture supernatants and that NE initially has access to only a few of the potential cleavage sites.

To date, it remains unclear what NE recognizes in its substrates. To this end, we tested the following hypotheses: First, that the specificity is encoded either by the primary, secondary or tertiary structure of the substrate. Second, that a specific domain in NE determines its substrates specificity.

3.1 The Specificity of NE for virulence factors is not encoded in NE substrates

3.1.1 A recognition motif for NE was not detected in the primary sequence of IpaB

To test the first hypothesis a series of *Shigella* IpaB mutants were tested for their susceptibility to NE cleavage (Guichon et al. 2001). These mutants contained individual deletions of 8-10 amino acids (aa) that spanned the coiled-coil region, as well as the putative transmembrane domains in the hydrophobic region of IpaB (figure 3.1). Coiled-coil motifs have been proposed as a common pattern for secreted virulence factors (Pallen et al. 1997; Miao et al. 1999) and present a possible recognition motif for NE. We reasoned that mutation in a particular NE recognition motif in IpaB should render the mutant protein resistant to NE cleavage.

To avoid false-positive results based on mis- or unfolded mutant proteins, we exclusively tested functional IpaB mutants. Since *Shigella* cannot invade or induce cytotoxicity in cells without a functional IpaB protein, we only tested IpaB mutants that were able to complement an *ipaB* deletion *Shigella* strain in epithelial cell invasion and macrophage cytotoxicity when introduced on a plasmid (Guichon et al. 2001). Furthermore, these IpaB

mutants were likely to be folded correctly as shown by limited proteolysis (Guichon et al. 2001).

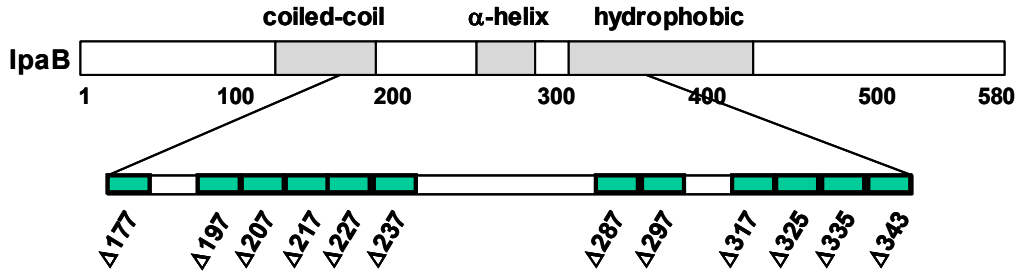


Fig. 3.1: Schematic representation of IpaB mutants.
Schematic presentation of the IpaB protein and the position of the mutations introduced (green rectangles). Adapted from (Guichon et al. 2001).

Supernatants of *S. flexneri* ΔipaB strains carrying the wt ipaB or the different mutant ipaB genes on a plasmid were collected and incubated with purified NE (pNE). IpaB is enriched in the supernatant because deletion of an *ipa* gene leads to a hypersecretory phenotype in *Shigella*. Although the cleavage patterns of the mutants were varying, none of the IpaB mutants was fully resistant to NE cleavage. Most mutants showed a similar cleavage pattern as the wildtype IpaB protein after treatment with pNE (figure 3.2). Interestingly, the IpaB mutant harboring the amino acid deletion Δ207-216, which is localized at the coiled-coil region, was even more susceptible to pNE cleavage than wildtype IpaB.

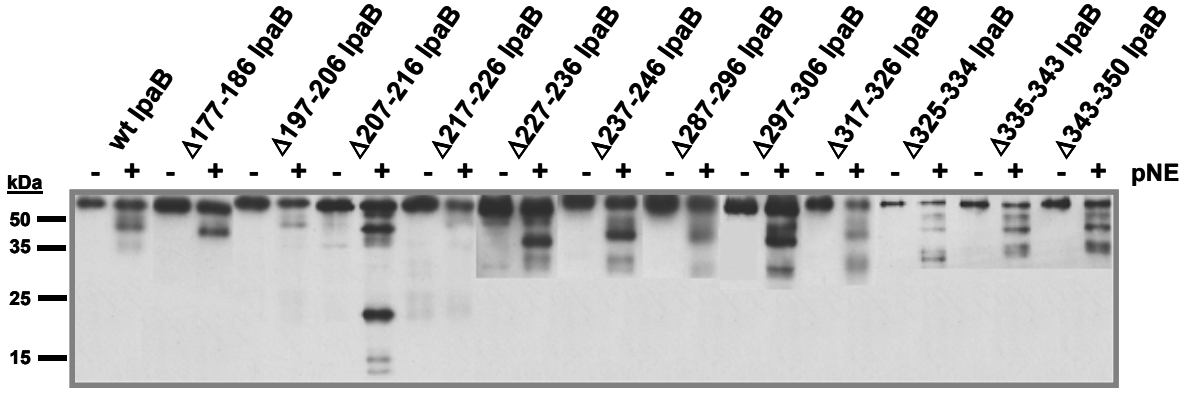


Fig. 3.2: The primary sequence of IpaB does not encode for NE specificity.
Supernatants of strains secreting wildtype or mutant IpaB protein were treated with pNE at a concentration of 100 ng/ml (+). As negative control buffer without pNE was added to each supernatant and the samples were treated equally (-). After 1 h incubation time, samples were TCA precipitated and analyzed by SDS-PAGE and immunoblotting using anti-IpaB antibody.

Taken together, this indicated that no exclusive NE recognition motif was present in these areas. Although the IpaA, B, C and D belong to different protein families, it could have been possible that NE recognizes a certain consensus sequence in these virulence factors. Therefore NE derived proteolytic products of IpaA, B and C were independently analyzed by MALDI-TOF mass spectrometry. However, such a consensus sequence was not detected (data not shown). From these experiments we concluded that the specificity of NE does not seem to be encoded in the primary sequence of NE substrates.

3.1.2 The secondary and tertiary structures of the substrate do not affect the specificity of NE for virulence factors

Next, we wanted to test whether higher order structures of the substrate are important for recognition and cleavage by NE. To this end, *Shigella* supernatant containing wildtype IpaB was heat-denatured and the NE cleavage assay was repeated. We observed that pNE was able to cleave native as well as denatured IpaB under the same conditions (figure 3.3; lanes 1, 2 and 6, 7). A previous study had shown that cathepsin G (CG) does not cleave *Shigella* virulence factors despite its high degree of homology to NE (Weinrauch et al. 2002). Therefore we simultaneously assessed if IpaB could be rendered susceptible to CG cleavage upon denaturation. However, purified CG (pCG) neither cleaved native nor denatured IpaB (figure 3.3; lanes 3,4 and 8, 9).

These experiments indicated that secondary or tertiary structures of the substrates are not mandatory for NE cleavage.

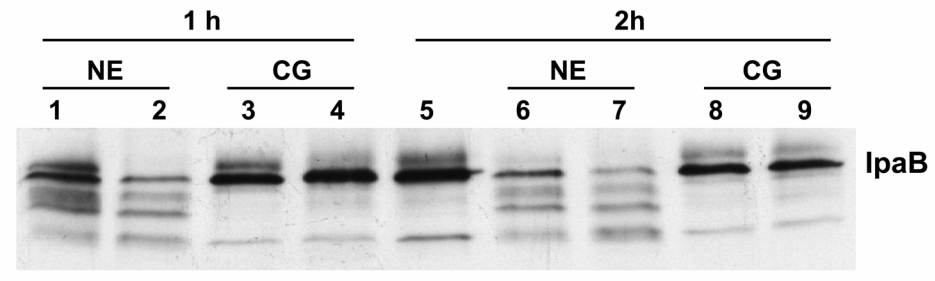


Fig. 3.3: NE but not CG degrades native and denatured IpaB.
Supernatant of *Shigella* (*M90T*ΔipaB + pUC19/wt ipaB) was incubated with pNE and pCG at 100 ng/ml for 1h (lane 1-4) and 2h (lane 6-9). Aliquots of the same supernatant were heat-treated (95°C, 10 min, followed by fast cooling) prior to incubation with pNE or pCG for 1 h (lane 2, 4) and 2 h (lane 7, 9). As negative control, buffer was added to heat-treated supernatant for 2 h (lane 5). Samples were analyzed by SDS-PAGE and immunoblotting using an IpaB antibody.

3.2 The Specificity of NE for virulence factors is encoded in NE

Since we were not able to detect a recognition motif for NE in the primary or higher order structures of the NE substrate IpaB, we addressed the question of NE specificity by a structural-functional analysis of NE. We reasoned that a recognition motif for virulence proteins existed in NE and that this motif could be mutated without comprising the catalytic activity of the protease. More specifically, we hypothesized that NE mutants could exist that were still active towards its peptide substrate but would no longer recognize and cleave virulence factors like IpaB.

To this end, we selected single or multiple amino acids for mutation in NE that could present such a NE recognition motif. We based this selection on a structural comparison of NE and CG, because CG does not cleave virulence factors despite its high degree of homology to NE. We planned to compare these NE mutants to wildtype NE in their ability to degrade the NE peptide substrate and *Shigella* virulence proteins. For this purpose, we first had to establish an expression system that was able to yield active recombinant NE on a consistent basis. In a recent study, active recombinant NE was transiently expressed in eukaryotic rat basophilic leukemia cells (RBL-1 cells) (Li and Horwitz 2001). However, we planned to express and purify recombinant NE in large amounts. Therefore we tried to express NE in bacteria and other non-mammalian cell systems, but we were unable to obtain active NE (see appendix 5.1). Thus, we switched to the aforementioned mammalian cell line and were finally able to express recombinant and active NE.

3.2.1 Expression of recombinant wildtype NE in mammalian cells

As mentioned above, active recombinant NE had been transiently expressed in RBL-1 cells (Li and Horwitz 2001). The cells had been transfected with the cDNA encoding for the full-length NE protein. Since N-terminally unprocessed NE is not active, these cells likely process the full-length NE to its mature form (Li and Horwitz 2001). Additionally, the RBL-1 cells properly targeted the enzyme to granules for storage, which is comparable to neutrophils (Gullberg et al. 1994; Gullberg et al. 1995).

We confirmed the transient expression of active recombinant NE using the aforementioned system (figure 3.4). The activity of NE was tested using a NE specific four amino acid peptide substrate coupled to a chromophore at the P1 position. Cleavage by NE results in the release of the chromophore leading to an increase in the optical density (OD) when

measured at 410 nm wave-length. NE activity was only observed in the lysate from cells expressing NE but not in the lysate of the negative control.

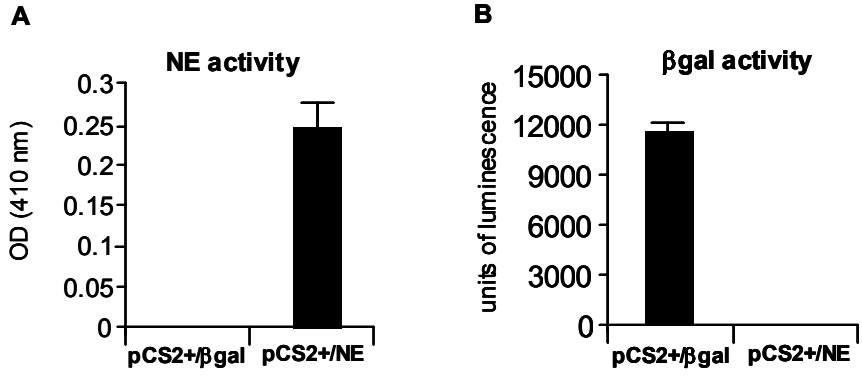


Fig. 3.4: Expression of active recombinant NE in transiently transfected cells.
RBL-1 cells were transfected with an expression plasmid containing the cDNA encoding for the full-length NE protein (pCS2+/NE) or with an expression plasmid carrying the ß-galactosidase gene of *E.coli* (pCS2+/ßgal) as negative control.
(A) NE activity of cell lysates from cells transfected with pCS2+/NE or with pCS2+/ßgal. The NE peptide substrate was added to the lysates and incubated for 30 min. The OD was measured at 410 nm wave-length. Lysate of non-transfected cells was treated identically and was used for normalization. All lysates were equivalent to $8x10^6$ cells. **(B)** ß-galactosidase activity of the same cell lysates ($1,4x10^4$ cell equivalents). The ß-galactosidase activity was measured according to the protocol described in chapter 2.5.1.

The fact that NE could be obtained in an active form proved RBL-1 cells to be a suitable tool for NE expression. However, for the upcoming experiments it was necessary to have access to a constant reservoir of recombinant NE. Therefore a stable NE expressing cell line was generated. Since the expression vector used for the transient expression of NE did not carry an antibiotic resistance gene, the DNA encoding for the NE full-length protein was subcloned into a suitable expression vector (pcDNA3) and this construct was used to stably transfect RBL-1 cells.

The transfected plasmid can randomly integrate into the genome of the host cell. This can result in varying expression levels of a plasmid-encoded gene depending on the chromatin context of the integration site. Therefore we tested the lysates of different single-cell derived cell lines carrying the NE gene for their ability to cleave the NE specific peptide substrate (figure 3.5). Of these clones, we selected the cell line derived from clone number four because its lysate exhibited the highest NE activity. The cells did not contain endogenous NE activity since lysate from cells transfected with the empty expression vector (vector lysate) did not cleave the peptide substrate. In contrast, addition of purified NE (pNE) to vector lysate lead to high activity confirming that NE is active under the experimental

conditions. Thus, we had established stable expression of active recombinant wildtype NE, which will be designated "wt" in the upcoming experiments.

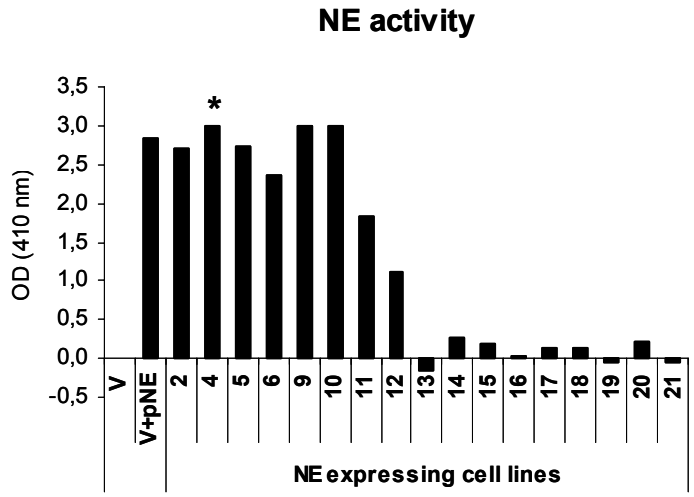


Fig. 3.5: Expression of active recombinant wildtype NE in stably transfected RBL-1 cells.
NE activity of lysates from 17 single-cell derived cell lines expressing NE or carrying the empty expression vector. The NE peptide substrate was added to the lysates and the OD was measured at 410 nm wave-length following a 30 min incubation period. All samples, including vector lysate that had been incubated with the substrate, were normalized against a vector lysate that had been incubated with the buffer of the substrate. As positive control, 1 μg pNE was added to vector lysate prior to addition of the substrate (V+pNE). All lysates were equivalent to $1x10^8$ cells. Cell line number 4 is marked with an asterisk and was used for subsequent experiments.

3.2.2 Kinetics of recombinant wildtype NE

Since whole cell lysates were used to compare the activities of wildtype and mutant NE, it was possible that the recombinant NE, although active, showed altered reactivity in this lysate background. Therefore we compared the kinetics of recombinant NE in the lysate (wt lysate) to that of purified NE (pNE).

The kinetics were tested using the same NE peptide substrate as in the previous experiments. By monitoring the OD at a wave-length of 410 nm every 30 sec over a 3 min time period, we assessed the time course of the cleavage of the substrate. The measurement started after addition of the lysate or pNE to the assay buffer containing the substrate.

We showed that wt lysate, equivalent to 4,2 x 10^6 cells, cleaved the NE peptide substrate with linear kinetics. Additionally, its substrate turnover rate was comparable to pNE at an approximate concentration of 350 ng/ml (figure 3.6). In contrast, lysate from cells

transfected with the empty expression vector (V lysate) did not degrade the peptide substrate. These data showed that the reactivity of the recombinant NE was not altered in the cell lysate.

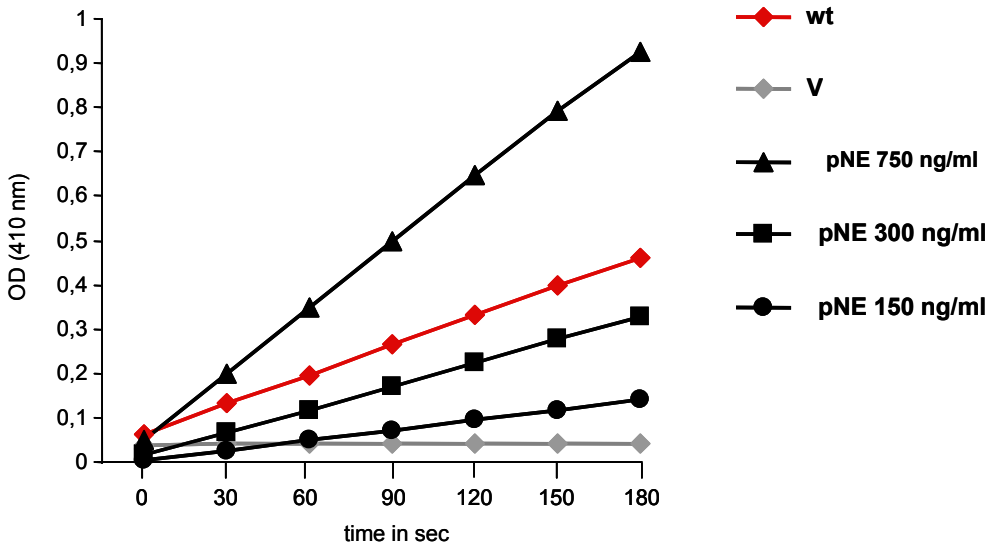


Fig. 3.6: Kinetics of recombinant wildtype NE.
NE kinetics of wt and vector lysate (wt and V respectively) compared to pNE at different concentrations. All lysates were equivalent to 4,2 x 10^6 cells and pNE was tested at concentrations of 150, 300 and 700 ng/ml. Upon addition of the samples to the assay buffer containing the NE peptide substrate, the OD was recorded every 30 sec for 3 min at a wave-length of 410 nm.

3.2.3 Specificity of recombinant wildtype NE

After we had established that the reactivity of the recombinant NE towards the NE peptide substrate was comparable to that of purified NE (pNE), we wanted to test the ability of recombinant NE to cleave IpaB in *Shigella* supernatant. As we had shown earlier, pNE did cleave IpaB at low concentrations of 100 ng/ml (see figure 3.3).

To compare the ability of recombinant and purified NE to cleave IpaB, we used identical concentrations of active protein. Since the amount of recombinant NE in the cell lysate could vary at each cell lysis, we used an indirect method to determine the amount of active protein. First, we measured the kinetics of pNE and wt lysate within a three minute time course experiment. Because the recombinant and the purified protein both showed linear kinetics, we were able to calculate NE activity units as increase in OD per minute (OD/min). We compared the NE activity units of wt lysate to that of pNE at a defined concentration. By doing so we were finally able to deduce the amount of wt lysate that corresponded to a given NE concentration.

In the presented experiment we determined a value of 0,12 NE units for pNE at a concentration of 500 ng/ml (figure 3.7a+b). This corresponded to 0,024 units for a concentration of 100 ng/ml pNE. In contrast, wt lysate at a concentration of 500 µg total protein per ml showed an activity of 0,02 NE units (figure 3.7a+b). Therefore, we used 0,024 NE units of wt lysate and compared it to 100 ng/ml of pNE. Consequently, we added 625 µg total lysate protein to each ml of *Shigella* supernatant to test cleavage of IpaB by the recombinant protein.

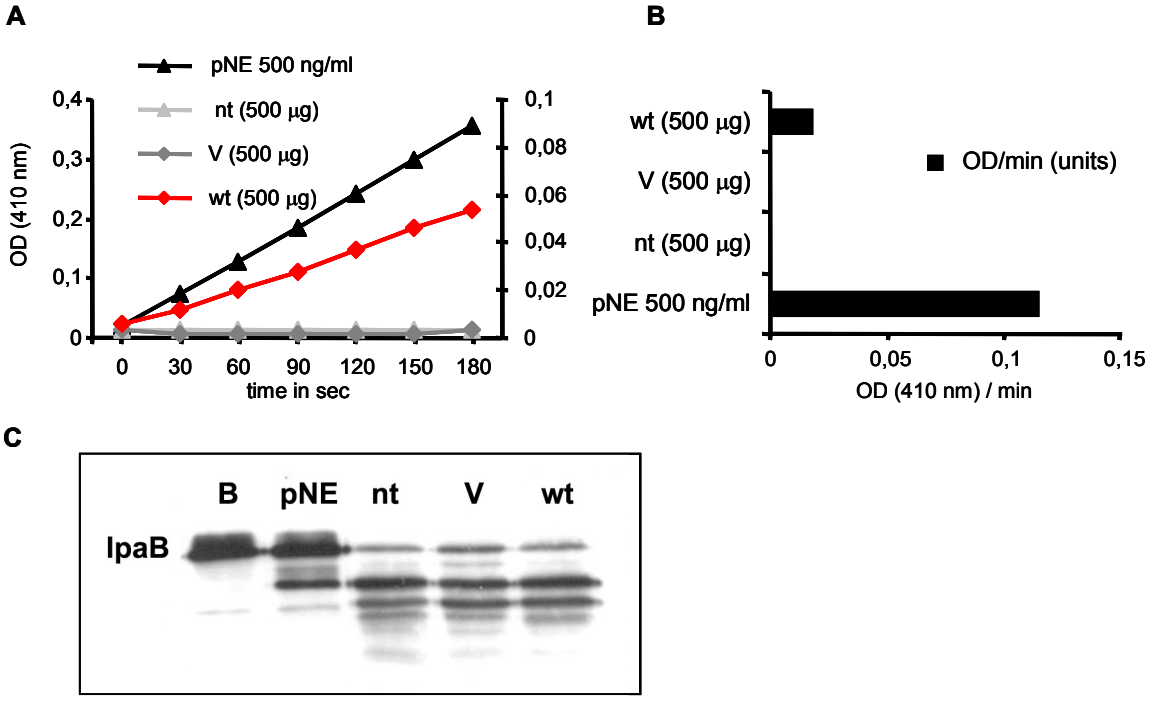


Fig. 3.7: Degradation of IpaB by lysates from RBL-1 cells.
(A) NE kinetics of wt and vector lysate (wt and V respectively), of lysate from non-transfected (nt) cells, and of pNE at a concentration of 500 ng/ml. The concentration of the lysates corresponded to 500 µg/ml total protein. Upon addition of the lysates or pNE to the assay buffer containing the NE peptide substrate, the OD was recorded every 30 sec for 3 min at a wave-length of 410 nm. The data for pNE was plotted against the left y-axis whereas the data of the lysates were plotted against the right y-axis. **(B)** NE activity units (OD/min) of the lysates and pNE calculated as change in OD/min. The units are based on the kinetic data measured in (A). **(C)** Supernatant of *Shigella* secreting wildtype IpaB was incubated with wt, V and nt lysate at a concentration of 625 µg/ml total lysate protein and with pNE at a concentration of100 ng/ml for 2 h. As negative control buffer without pNE was added to supernatant. Samples were TCA precipitated and aliquots were analyzed by SDS-PAGE and immunoblotting using an anti-IpaB antibody.

Unexpectedly, IpaB was not only cleaved by pNE and lysate expressing the recombinant NE but also by lysates of the negative controls (figure 3.7c). This indicated that the RBL-1 cell line itself contained an IpaB degrading activity. RBL-1 cells are basophils and thus

granulocytes. Therefore they store many proteases such as tryptase, chymase or carboxypeptidase in their granules (Marone et al. 1997). Obviously these proteases were present in the cell lysates and degraded IpaB. To be able to measure the specificity of the recombinant NE in the cell lysate, we had to inhibit the endogenous RBL proteases without affecting the activity of the recombinant NE.

It was not possible to use commercially available protease inhibitor cocktails because all of them contain general serine protease inhibitors like PMSF that also block NE. Therefore we designed an individual mixture of general and specific protease inhibitors. The general inhibitors blocked complete protease families except serine proteases, e.g. cystein- or metalloproteases, whereas the specific inhibitors suppressed, for example, chymotrypsin-like but not elastase-like serine proteases (for composition of the IC see chapter 2.5.2). First, we tested if this inhibitor cocktail (IC) affected the NE activity of the recombinant or the purified NE. Therefore we measured their kinetics in the absence and in the presence of the IC at different concentrations.

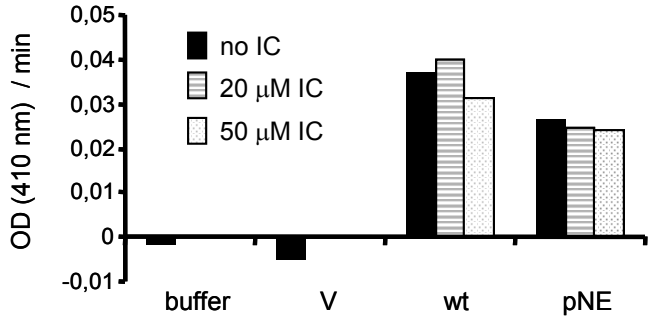


Fig. 3.8: NE activity units of wt lysate and pNE in the presence or absence of the inhibitor cocktail (IC).
NE activity units of buffer, wt and vector lysate (wt and V respectively), and of pNE (70 ng/ml). The lysates were equivalent to 1,7 x 10^6 cells. The IC (20 µM and 50 µM) and the solvent of the IC were added to the lysates or pNE and incubated at RT for 15 min. The samples were added to the assay buffer containing the NE peptide substrate and the kinetics were measured by reading the OD at 410 nm wave-length every 30 sec over 3 min. The calculated NE units are based on the kinetic data and represent the change in absorbance per minute.

We observed that the inhibitor cocktail at both concentrations did not alter the NE activity of pNE (figure 3.8). However, the NE kinetics of the wt lysate were slightly decreased at an IC concentration of 50 µM but not at 20 µM. Therefore we tested whether the IC at a concentration of 20 µM was able to block the unspecific IpaB degradation by the endogenous cell proteases. To this end, we added the IC to the cell lysates prior to

incubation with the IpaB containing *Shigella* supernatant. Since we wanted to test the effects of the IC at maximal lysate concentrations, equal cell equivalents were added to the supernatant independent of the lysates' activity units.

In fact, the IC at a concentration of 20 µM almost completely blocked the degradation of IpaB by endogenous RBL-1 proteases (figure 3.9, lane 4), while it did not inhibit IpaB cleavage by pNE added to vector lysate (figure 3.9, lane 5), implying that the inhibitor cocktail did not interfere with the IpaB cleaving activity of pNE. In addition, wt lysate did also cleave IpaB in the presence of the inhibitor cocktail (figure 3.9, lane 6). This proved the specificity of the recombinant wildtype NE towards the virulence factor IpaB.

Since endogenous RBL proteases were not completely inhibited by the IC at 20 µM, the concentration was increased to 50 µM in all subsequent experiments. At this concentration the inhibitor cocktail also did not interfere with the IpaB cleaving activity of purified or recombinant NE (see figure 3.16).

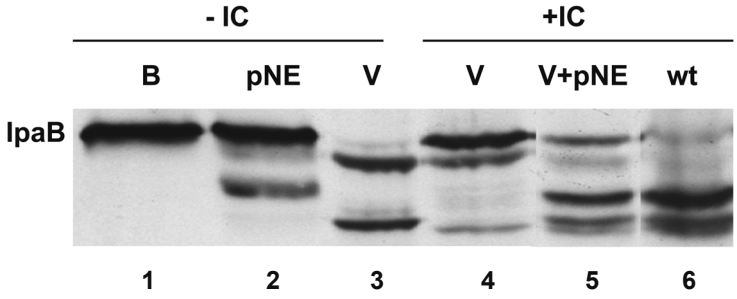


Fig. 3.9: The inhibitor cocktail (IC) can block degradation of IpaB by endogenous proteases without inhibiting NE specificity.
Supernatant of *Shigella* secreting wildtype IpaB was incubated with vector lysates in the presence or absence of the IC, and with wt lysate and vector lysate that contained pNE (20 ng/1x10^6 cell equivalent) in the presence of the IC. As controls, supernatant was also incubated with buffer (B; lane 1) and pNE in buffer (100 ng/ml) (lane 2). All lysates were equivalent to 7x10^6 cells. The IC (20 µM) or the solvent of the IC were added to the samples and incubated at RT for 15 min prior to addition to the supernatant. The reaction mixtures were incubated for 2 h at 37°C. After protein precipitation, aliquots were analyzed by SDS-PAGE and subsequent immunoblotting using an anti-IpaB antibody.
Buffer (lane 1), pNE in buffer with solvent of IC (100 ng/ml) (lane 2), vector lysate with solvent of IC (lane 3), vector lysate with IC (lane 4), vector lysate + 140 ng/ml pNE with IC (lane 5), and wt lysate with IC (lane 6).

At this point, we had established the expression of active recombinant wildtype NE and were able to assess its specificity for the *Shigella* virulence factor IpaB. As a next step, we designed the NE mutants and expressed them in order to compare their activities and specificities to wildtype NE.

3.2.4 Design of NE mutants

As mentioned earlier, the proteases NE and CG share many attributes and display a striking similarity in their crystal structures. When superimposed, the α-carbons of NE and CG only display a root mean square deviation (RMSD) of 0,9 Å (see chapter 1, figure 1.8). Since a RSMD value of zero means that structures are identical in conformation (Maiorov and Crippen 1994), it is apparent that the crystal structures of NE and CG are extremely similar (Bode et al. 1986b; Hof et al. 1996). Yet, NE cleaves virulence factors whereas CG does not. Thus, there had to be subtle differences that determine the opposing specificities. Therefore we examined both structures using the programs PYMOL (DeLano 2002) and the SWISS-PDB VIEWER (Guex 1997). Indeed, we identified single amino acids or stretches of multiple amino acids that were significantly different in NE and CG (figure 3.11). These residues were mainly located in the substrate-binding cleft formed by the ß-barrel domains of the enzymes. Interestingly, most of these amino acids were part of the previously described NE binding pockets (figure 3.10) (Bode et al. 1986b; Bode et al. 1989).

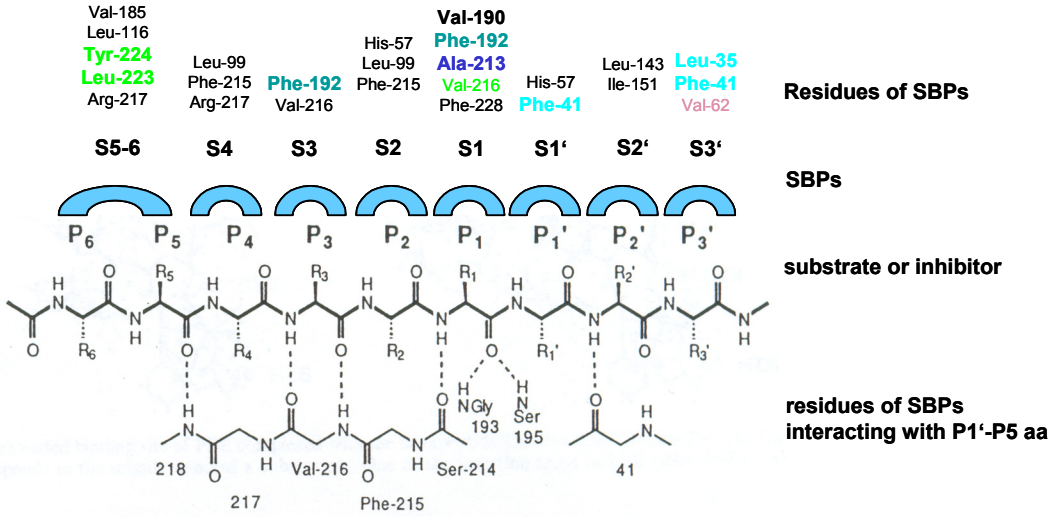


Fig. 3.10 Schematic representation of the NE substrate binding pockets (SBP).
Schematic representation of the NE substrate binding pockets (S_6-S_3') that interact with the residues of a NE substrate or inhibitor (P_6-P_3'). The carbonyl group of the scissile bond forms hydrogen bonds with the residues of the oxyanion hole (Gly 193 and Ser 195). The residues composing the pockets are presented in the 3-letter code and their position within NE is indicated [according to chymotrypsinogen numbering (Hartley B.S. 1971)]. Some of these amino acids were mutated and tested in this study. They are highlighted in the colour corresponding to figure 3.11. Significant residues that had been suggested to influence the specificity of NE are shown in bold. This presentation is based on the crystallization of NE with the inhibitor TOM (Bode et al. 1986b) and adapted from (Bode et al. 1989)

Since CG does not cleave *Shigella* virulence factors, we assumed that replacement of the differing amino acids in NE with their structural CG counterparts might transfer the CG specificity onto the NE mutants. Consequently, NE harboring these mutations should not cleave the *Shigella* virulence factors any longer, but still be active towards the peptide substrate.

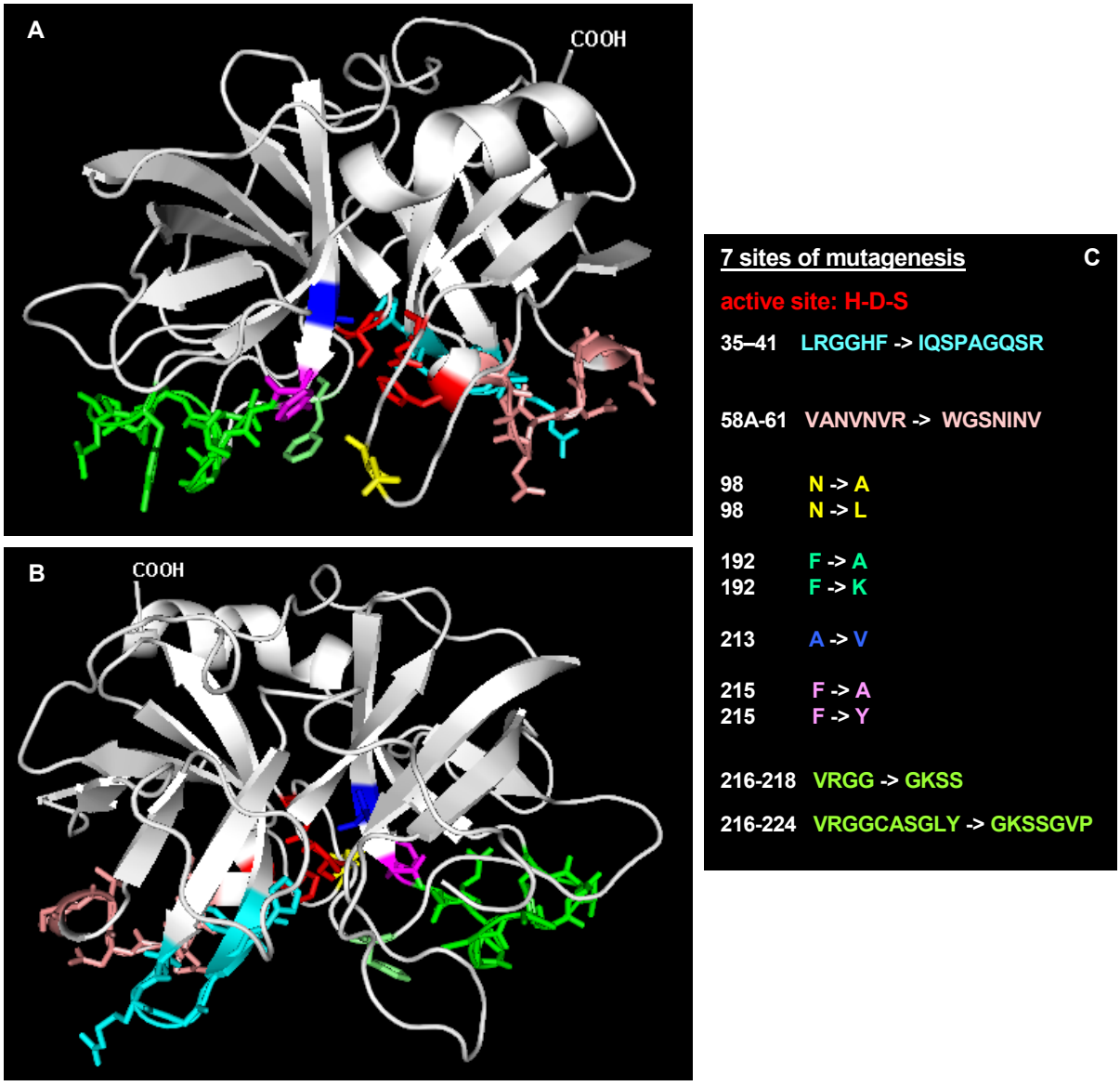


Fig. 3.11: NE mutants and localization within the NE structure.
NE was mutated by single or multiple amino acid exchanges at seven different sites. **(A)** Front view of the NE structure (Wei et al. 1988) depicted as cartoon [PYMOL (DeLano 2002)]. The residues that were mutated are shown as sticks and the different colors correspond to the different mutations indicated in (C). For orientation the C-terminus is marked (COOH). **(B)** Picture (A) rotated by 180^0C. **(C)** Position of the NE residues in the sequence [numbering according to chymotrypsin (Hartley B.S. 1971)]. The residues that were exchanged or introduced are shown in the single letter code.

We exchanged four single amino acids and four amino acid stretches in NE with their structural CG counterparts. To test the influence of a non-polar and not charged residue at the positions 98, 192 and 215, we additionally exchanged these amino acids with an alanine. The mutations were introduced by site-directed mutagenesis in the NE gene encoded on the plasmid pcDNA.3/NE. The eleven NE mutants that were generated and their location within the NE structure are shown in figure 3.11.

3.2.5 Analysis of NE mutants – Cleavage of peptide substrates

RBL-1 cells were stably transfected with the 11 different pcDNA3/NE mutant constructs. As for the establishment of the cell line expressing recombinant wildtype NE (wt), a number of different single-cell derived cell lines for each mutant were tested for their NE activity (see appendix, chapter 5.2). Interestingly, we only observed NE activity in cell lines of seven NE mutants (35-41, N98L, F192A, F192K, A213V, F215A and F215Y). Among those only mutant F215A showed NE activity that was lower than the activity of wt NE. Of these seven mutants we selected the cell line whose lysate showed the highest NE activity per cell number for subsequent analysis.

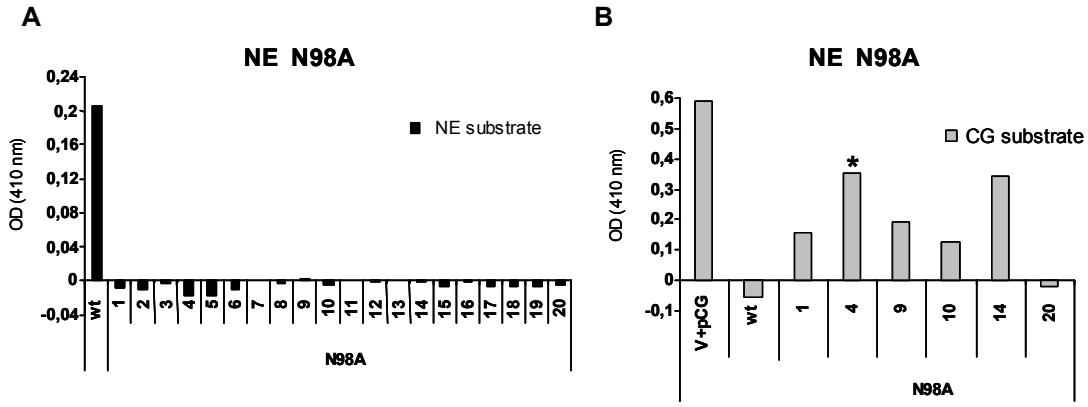


Fig. 3.12: NE and CG activity of different cell lines expressing NE mutant N98A
(A) NE activity of lysates from 20 single-cell derived cell lines expressing NE mutant N98A. The NE peptide substrate was added to the lysates and the OD was measured at 410 nm wave-length after a 30 min incubation period. As positive control wt lysate was used. **(B)** CG activity of lysates from 6 single-cell derived cell lines expressing NE mutant N98A. Activity was measured as in (A) but the CG instead of the NE peptide substrate was used. As positive control vector lysate containing 1 µg pCG (V+pCG) was used. Clone number, marked with an asterisk, was chosen for subsequent experiments.
All samples were normalized against a vector lysate that had been incubated with the respective substrate. All lysates were equivalent to $1x10^5$ cells and treated equally.

The remaining four mutants 58A-61, N98A, 216-218 and 216-224 did not exhibit NE activity, although more than 16 single-cell derived cell lines for each mutant were tested (N98A as representative mutant is shown in figure 3.12a). This indicated that the introduced amino acids had possibly changed the specificity of the mutants for the peptide substrate. Since the introduced residues were part of the CG protein, we assumed that they had introduced a CG-like activity into these NE mutants. Therefore we assessed the ability of several cell lines of these NE mutants to cleave the CG peptide substrate. The CG peptide is identical to the NE peptide substrate except for a N-terminal modification and the P1 amino acid, which is valine in NE and phenylalanine in CG. The CG activity and kinetics were measured in an identical experimental set-up as for NE. However, RBL-1 cells contained endogenous activity against the CG peptide substrate. Therefore it was important to normalize the samples against the equal amount of vector lysate that had been incubated with the CG peptide for the identical amount of time. We observed that lysates from cells expressing NE mutants 58A-61, N98A, 216-218 and 216-224 indeed did cleave the CG peptide substrate [see appendix, chapter 5.2; (N98A as representative mutant is shown in figure 3.12b)]. Again, we selected the cell line with the highest CG activity of each mutant for further experiments.

To confirm the NE- or CG-like activities of lysates from the different mutants, we re-tested the cell lysates of all mutants for their ability to degrade the NE and the CG peptide substrate (figure 3.13). For this comparison we used the cell line of each mutant that had exhibited the highest activity towards the respective substrate. As expected, the lysates of mutants 58A-61, N98A, 216-218 and 216-224, which had cleaved the CG but not the NE substrate in the previous experiments, exclusively cleaved the CG peptide substrate. Thereby we reconfirmed the observed high activity of the lysate of mutant 216-218. In contrast, the lysates of the mutants 35-41, N98L, F192K, A213V and F215Y exclusively cleaved the NE peptide substrate. Interestingly, two mutants, F192A and F215A, cleaved both the NE and the CG substrate, albeit with different activities. Lysate from cells expressing NE F215A showed low activity towards both substrates. However, lysate from cells expressing NE F192A preferentially cleaved the NE peptide substrate. Taken together, five of the eleven mutants exclusively cleaved the NE peptide substrate, while four exclusively cleaved the CG peptide substrate. Two mutants cleaved both substrates.

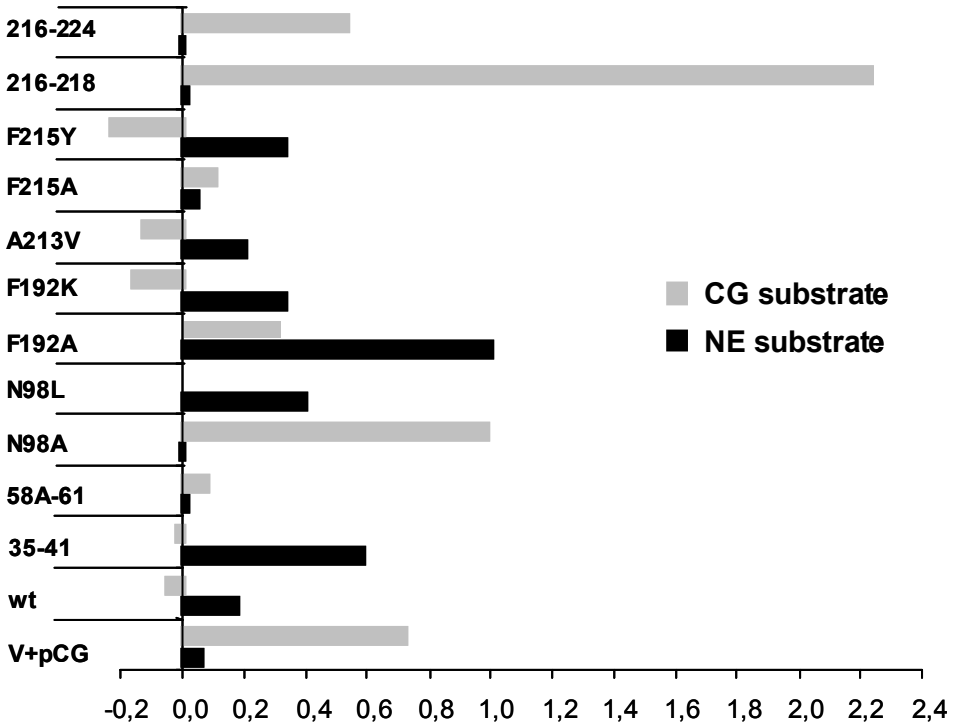


Fig. 3.13: NE and CG activity of the different NE mutants.
NE or CG activity of lysates from cells expressing the different NE mutants. The NE (black) and CG (grey) peptide substrates were individually added to the lysates. The OD was measured at 410 nm wave-length after a 30 min incubation period. The samples were normalized against vector lysate that had been incubated with the respective substrate. As positive controls, the activities of wt lysate or of vector lysate containing 1 μg pCG (V+pCG) were measured. All lysates were equivalent to $1x10^5$ cells and treated equally. The data represent one of five independent experiments.

We reasoned that specific inhibition of the activities of wt NE and of the different mutants would confirm that the observed substrate cleavage was caused by the recombinant proteins and not by the lysate. Therefore we assessed the abilities of the recombinant proteins to cleave the NE or CG peptide substrate in the presence or absence of specific NE and CG inhibitors. The inhibitors used in this study are both irreversible inhibitors. The NE inhibitor is identical to the peptide substrate except for the chromophore, which is replaced by a chloromethyl ketone (CMK) group. NE binds the inhibitor but its active triad cannot cleave the peptide bond of the P1 amino acid and the CMK group. Thus the enzyme inhibitor complex does not dissociate. The CG inhibitor acts in a similar way and is a three amino acid peptide with a benzyloxycarbonyl group at the N-terminus and a CMK group at the C-terminus. Like NE, CG binds this inhibitor and is incapable of cleaving the peptide bond between the P1 amino acid phenylalanine and the CMK.

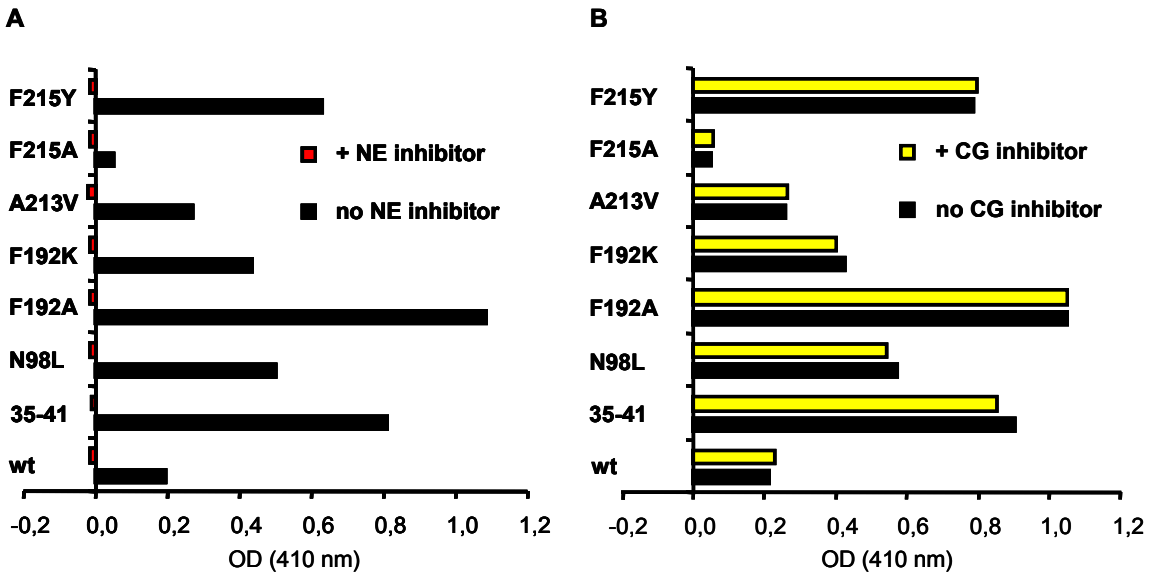


Fig. 3.14: NE activity of wt and NE-like mutants in the presence or absence of NE or CG inhibitors.
NE activity of lysates from cells expressing wt, the NE mutants that exclusively cleaved the NE peptide substrate, or the two mutants that cleaved both substrates. The activity was measured in the absence and in the presence of **(A)** the NE inhibitor (NE-CMK, 200 μM (red)) or **(B)** the CG inhibitor (Z-GLF-CMK, 500 μM (yellow)). Inhibitor and lysates were incubated at RT for 15 min prior to addition of the NE peptide substrate. The OD was measured at 410 nm wave-length after a 30 min incubation period. Samples were normalized against vector lysate. All lysates were equivalent to $1x10^5$ cells and treated equally. The data represent one of three independent experiments.

We observed that the NE activity of lysates from cells expressing wt or the seven NE mutants that cleaved the NE peptide substrate (35-41, N98L, F192A, F192K, A213V, F215A and F215Y) was blocked by the NE but not by the CG specific inhibitor (figure 3.14). The NE inhibitor completely suppressed the activity of the recombinant proteins independently of the different activity levels of the proteins. In contrast, the CG inhibitor did not affect the NE activities of these mutants and of wt NE, although it was used at a higher concentration than the NE inhibitor. This proved that the observed activity of wt and of the mutants was specific to the recombinant proteins.

In a second experiment, we analyzed the CG activity of the NE mutants 58A-61, N98A, F192A, F215A, 216-218 and 216-224 in the presence of the NE or CG inhibitor (figure 3.15). The CG activity of the lysates from cells expressing the mutants 58A-61 and F215A was blocked completely by the CG but not by the NE inhibitor. In contrast, the CG activity of the N98A, F192A and 216-224 lysates were affected by the CG inhibitor but not completely blocked. The NE inhibitor did only weakly interfere with the CG activity of

these mutants. Unexpectedly, the CG activity of the lysate from cells expressing the mutant 216-218 was impaired to a stronger degree by the NE than by the CG inhibitor. However, since the CG activity of this lysate was extremely high, we could only observe an inhibitory effect when the lysate was diluted.

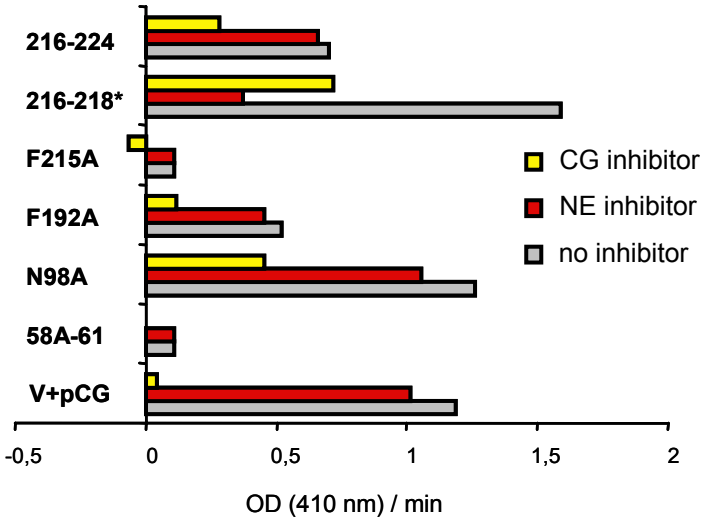


Fig. 3.15: CG activity of CG-like mutants in the presence or absence of NE or CG inhibitors.
CG activity of lysates from cells expressing the NE mutants 58A-61, N98A, F192A, F215A, 216-218 and 215-225. The activity was measured in the absence and in the presence of the CG inhibitor (Z-GLF-CMK, 1 mM (yellow)) or the NE inhibitor (NE-CMK, 1 mM (red)). Inhibitors and lysates were incubated at RT for 15 min before addition of the CG peptide substrate. The OD was measured at 410 nm wave-length after 30 min incubation period. Samples were normalized against vector lysate. As positive control, 1 μg pCG was added to vector lysate (V+pCG). Except for 216-218*, marked with an asterisk, all lysates were equivalent to $1x10^5$ cells. The lysate of 216-218 was equivalent to $3,3x10^4$ cells. All lysates were treated equally. The data represent one of three independent experiments.

3.2.6 Analysis of NE mutants – Cleavage of Shigella virulence factors

In the previous experiments, we had observed that most mutants exclusively cleaved either the NE or the CG peptide substrate. Only two mutants cleaved both substrates, although one of them, F192A, preferentially cleaved the NE peptide substrate. Next, we wanted to test the ability of these NE mutants to cleave *Shigella* virulence factors. We assumed that the mutants that cleaved the NE peptide substrate (NE-like mutants) would act as wt NE and cleave the virulent proteins. In contrast, the four mutants that exclusively cleaved the CG peptide substrate (CG-like mutants) should behave like CG and not cleave the virulence factors. First, we assessed the ability of the NE-like mutants to cleave IpaB in the presence of the inhibitor cocktail (IC). As positive controls, we used wt lysate and pNE that had been added to vector lysate. To add equal amounts of active protein to IpaB containing *Shigella*

supernatant, we determined the individual NE activity units of the recombinant and the purified proteins. However, we were unable to determine the kinetics of the lysates from cells expressing the mutants A213V and F215A within the three minute time course of the experiment (data not shown). This had not been expected because these mutants had cleaved the substrate after an incubation period of 30 minutes in an earlier experiment (figure 3.13). The mutants 35-41, N98L, F192A, F192K, and F215Y as well as wt NE and pNE did cleave the NE peptide substrate with linear kinetics and we could calculate the activity units per 1,6 x10^6 cell equivalents (figure 3.16a). We next assessed the ability of the mutants to cleave IpaB. To test different concentrations of the recombinant proteins we used mutant and wt lysates corresponding to three different activity units (0,01, 0,025 and 0,05 NE units).

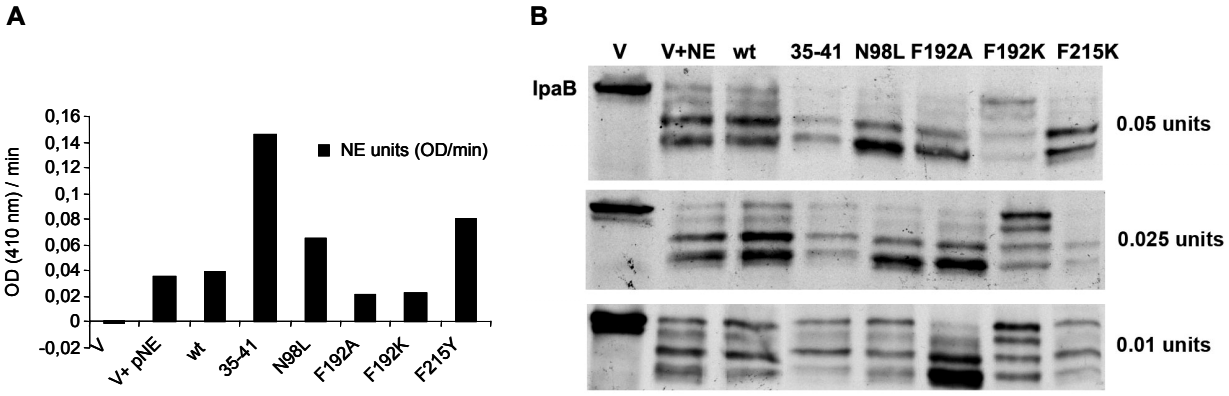


Fig. 3.16: NE mutants with NE activity cleave IpaB.
All lysates were tested in the presence of the IC (50 μM).
(A) NE activity units of lysates from cells expressing wt or the mutants 35-41, N98A, F192A, F192K, and F215Y. As second positive control next to wt, 1 μg pNE had been added to vector lysate (V+pNE; 100 ng/ 1x10^6 cell equivalents.). Vector lysate was used as negative control. All lysates were equivalent to 1,6 x10^6 cells and had been incubated with the IC (50 μM) at RT for 15 min before measurement. The NE kinetics were measured by reading the OD at 410 nm wave-length every 30 sec over 3 min. The calculated NE units represent the change in OD per minute.
(B) Supernatant (1ml) of *Shigella* secreting wiltype IpaB was treated with the different lysates corresponding to 0,05, 0,025 and 0,01 NE units in the presence of the IC. As negative control, vector lysate was added to the supernatant. In each experiment the amount of vector lysate corresponded to the highest cell equivalence used for the other lysates. The reaction mixtures were incubated for 2 h at 37°C. After protein precipitation, aliquots were analyzed by SDS-PAGE and subsequent immunoblotting using an anti-IpaB antibody.

We observed that all mutants cleaved IpaB in the presence of the IC even when low activity units were used (figure 3.16b). 0,01 NE units corresponded to a pNE concentration of 41 ng/ml, which was even lower than the concentration of 100 ng/ml used in our initial IpaB

cleavage experiments (see figure 3.3). Interestingly, the IpaB cleavage pattern upon treatment with the lysate of F192K mutant implied that the activity of F192K towards IpaB was slightly reduced as compared to the other mutants. Taken together, these five mutants showed the same specificity towards IpaB like wt NE.

We also wanted to test the ability of the other six mutants to cleave IpaB. Since we could not measure the NE kinetics of the CG-like mutants and of A213V and F215A, we had to determine the amount of lysate to test for IpaB cleavage in a different way. To this end, we assessed the kinetics of wt NE, calculated its activity units (figure 3.17a) and linked this data to the concentration of total protein in the respective lysate. Since we wanted to ensure that mutants with putatively low activity could potentially cleave IpaB, we tested high activity units in this experiment. 0,05 NE units of wt lysate corresponded to a concentration of 333 µg total protein. We used the same amount of total protein of the mutant lysates and vector lysate to test for IpaB cleavage. To prove that this method of determining the lysate is reliable, we used as positive controls the lysates of the NE mutants 35-41, N98L and F215Y that had cleaved IpaB in the previous experiment. Additionally, we used vector lysate containing pNE (V+pNE) that corresponded to 0,05 NE units as positive control for wt NE.

We observed that the lysate from cells expressing the NE mutant A213V did cleave IpaB just as the positive control lysates (figure 3.17b). In contrast, F215A and the CG-like mutants 58A-61, N98A, and 216-224 did not cleave IpaB. Interestingly, incubation of IpaB with mutant 216-218 resulted in negligible IpaB degradation, which does not compare to the IpaB cleavage pattern observed upon treatment with wt or NE-like mutants.

To confirm that the CG-like mutants and F215A were active although they did not cleave IpaB like wt NE, we tested the kinetics of these mutants at similar total protein concentration using the CG peptide substrate (figure 3.17c). It is obvious that the activities of 58A-61 and F215A were low, because their activity units were hardly higher than the activity units of vector lysate. However, N98A, 216-218 and 216-224 proved to be active since they showed a higher CG activity than the vector lysate. Again, the 216-218 lysate showed an extremely high CG activity that was higher than that of 5 µg/ml pCG.

Taken together, F192A and the NE mutants that exclusively cleaved the NE peptide substrate cleaved IpaB like recombinant or purified wt NE. In contrast, NE mutants that exclusively recognized the CG peptide substrate did not, or in the case of 216-218 did only slightly target IpaB. F215A, which degraded both substrates with low activity, did also not cleave IpaB.

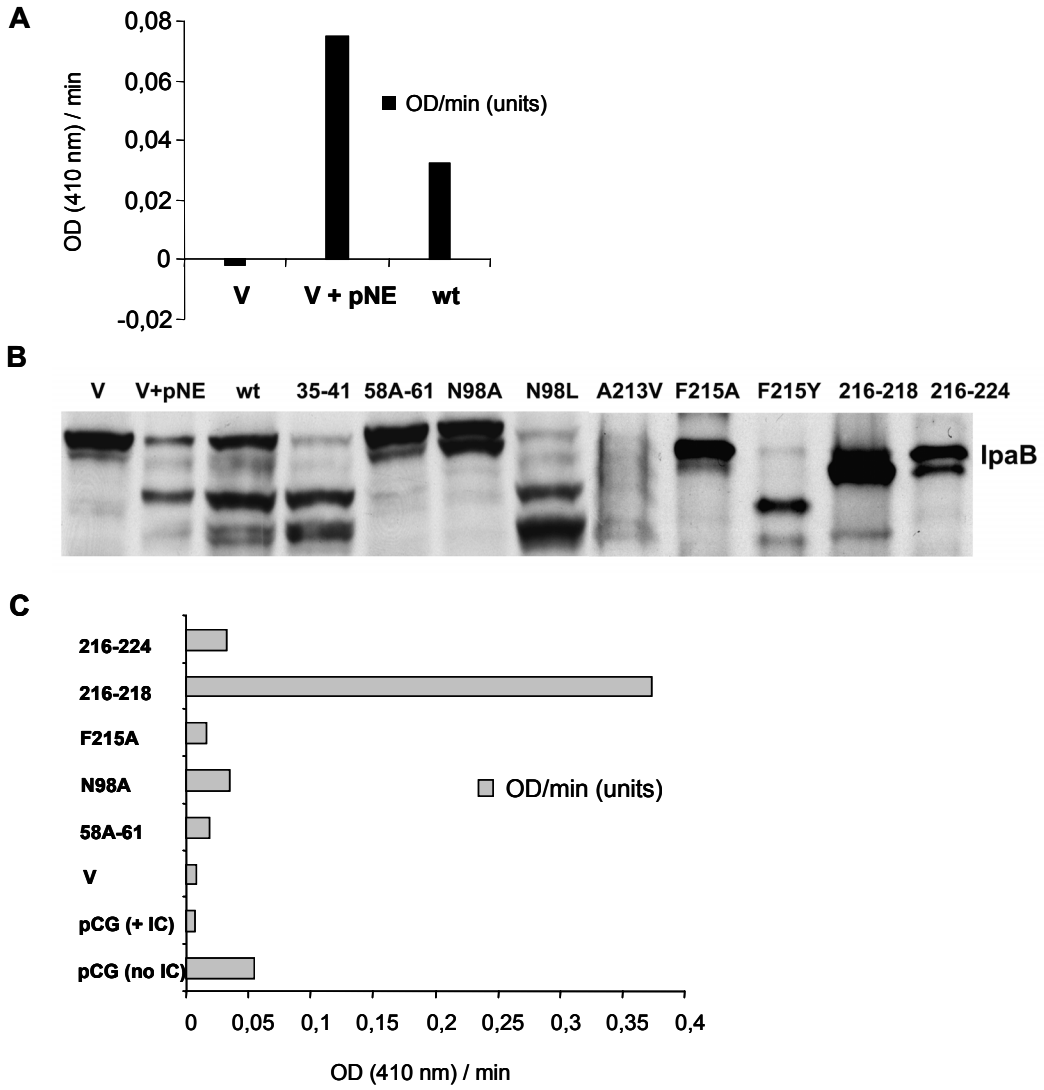


Fig. 3.17: IpaB is cleaved by NE-like but not by CG-like mutants.
All lysates were tested in the presence of the IC (50 μM).
(A) NE activity units of wt lysate, vector lysate containing pNE at a concentration of 175 ng/200 µg total protein (V+pNE), and vector lysate (V). All lysates were equivalent to 200 µg total protein. The NE kinetics were measured by reading the OD at 410 nm wave-length every 30 sec over 3 min. The calculated NE units represented the change in OD per minute. For wt lysate, 200 µg total protein corresponded to 0,03 NE units.
(B) Supernatant of *Shigella* secreting wildtype IpaB was coincubated with the different lysates at a total protein concentration of 333 μg/ml. This concentration corresponded in the wt lysate to NE activity units of 0,05 (see A). As negative control, vector lysate and, as positive control, V+pNE corresponding to 0,05 NE units were added to the supernatant. The reaction mixtures were incubated for 2 h at 37°C. After protein precipitation, aliquots were analyzed by SDS-PAGE and subsequent immunoblotting using an anti-IpaB antibody.
(C) CG activity units of vector lysate and of the lysates containing the mutants 58A-61, N98A, F215A, 216-218 and 216-224. All lysates were equivalent to 100 µg total protein. As positive control, the CG kinetics of pCG at a concentration of 5 μg/ml was measured in the presence (+IC, 50 μM) and absence of the IC (no IC). The CG kinetics were measured as in (A).

It has been shown that NE cleaves membrane-bound IcsA while it does not target membrane proteins that are important for *Shigella* homoeostasis, e.g. the outer membrane protein A (OmpA) (Weinrauch et al. 2002). Therefore, we tested the ability of wt and mutant NE to cleave IcsA and OmpA. We assumed that the wt NE and the NE-like mutants, including F192A, would target IcsA but not OmpA. This would prove that these recombinant proteins, just as pNE, only cleave virulence factors. To confirm that the IcsA cleavage was exclusively caused by the activity of the recombinant proteins, we tested the lysates of NE-like mutants, including F192A, and wt NE also in the presence of the specific NE inhibitor.
As in the previous experiment, we used identical total protein concentrations of the different lysates to test for IcsA and OmpA cleavage (figure 3.18a). However, in this experiment we used the total protein concentration of wt lysate corresponding to 0,01 NE units as reference because in a preliminary experiment we had observed that wt NE completely cleaved IcsA at 0,01 NE units.
We incubated *Shigella* with the cell lysates and analyzed the bacterial lysates for cleavage of the membrane-bound IcsA and OmpA. As expected, the wt NE and the NE-like mutants 35-41, N98L, F192A, F192K, A213V and F215Y cleaved IcsA but not OmpA (figure 3.18c). In addition, they did not cleave IcsA in the presence of the specific NE inhibitor. This proved that the recombinant proteins specifically targeted IcsA. In contrast, the mutants N98A and 216-224 did not cleave IcsA, despite being active (figure 3.18b). Interestingly, mutant 216-218 partially degraded IcsA. We speculated that this partial degradation was due to the high activity of the mutant, since 216-218 exhibited a much higher activity per total protein than the other mutants (figure 3.18c). Therefore we tested the ability of the lysate of 216-218 to cleave IcsA at a lower total protein concentration. At this concentration the activity units were comparable to the CG activity of mutant 216-224. In this experimental set-up NE mutant 216-218 did not target IcsA (figure 3.18d). Furthermore, the NE mutants 58A-61 and F215A did not show any CG activity and did not degrade IcsA.
In summary, we were able to compare the activities of recombinant wt and mutant NE towards synthetic and biological substrates. Like wt NE, the NE mutants 35-41, N98L, F192A, F192K, A213V and F215Y specifically cleaved the NE peptide substrate and the *Shigella* virulence factors IpaB and IcsA. In contrast, the NE mutants N98A, 216-218 and 216-224 that no longer cleaved the NE but the CG peptide substrate had lost their specificity for these virulence factors.

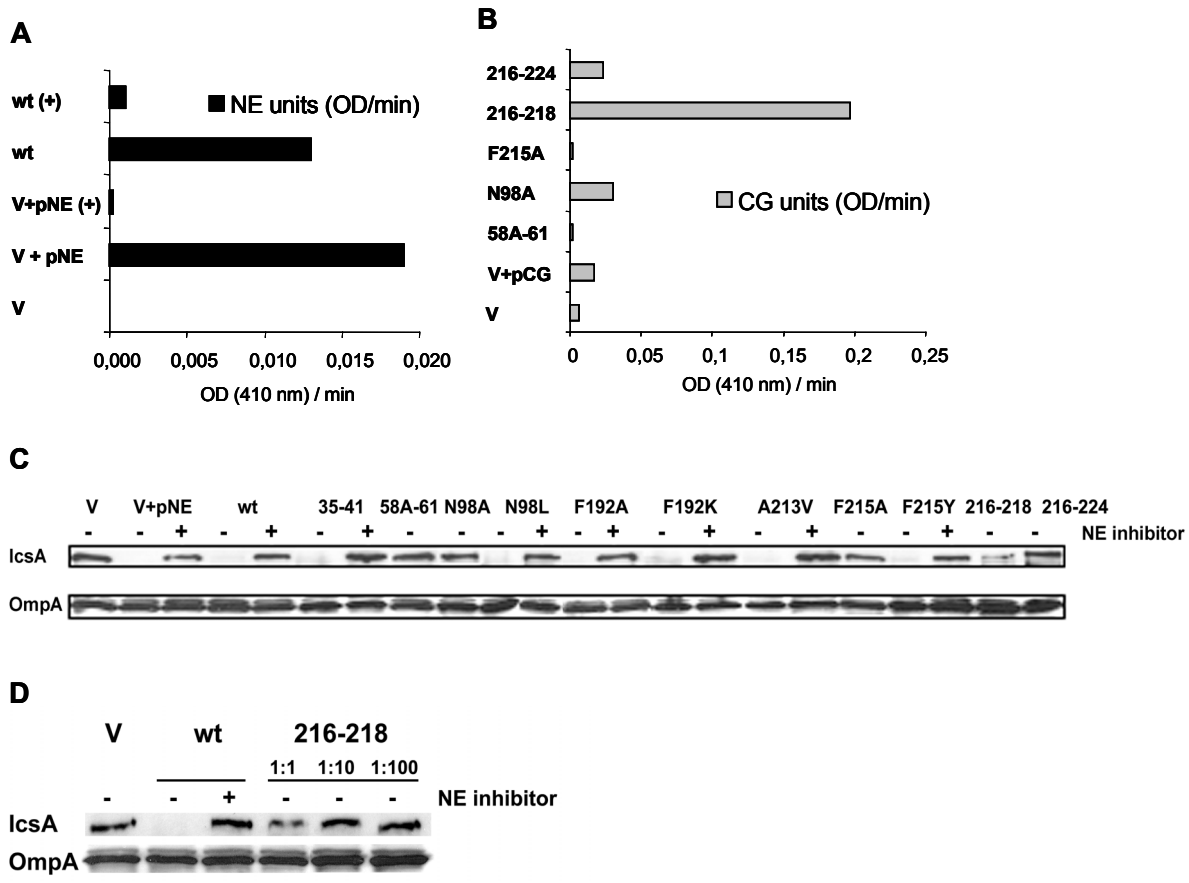


Fig. 3.18: IcsA is cleaved by NE-like but not by CG-like mutants.
All lysates were tested in the presence of the IC (50 μM).
(A) NE activity units of wt lysate, vector lysate containing pNE at 75 ng/100 µg total protein (V+pNE), and vector lyste (V) in the absence or presence (+) of the NE inhibitor (NE-CMK, 200 µM). All lysates were equivalent to 100 µg total protein. The NE kinetics were measured by reading the OD at 410 nm wave-length every 30 sec over 3 min. The calculated NE units represented the change in OD per minute. For wt lysate, 100 µg total protein corresponded to 0,013 NE units.
(B) CG activity units of vector lysate and of the lysates containing the mutants 58A-61, N98A, F215A, 216-218 and 216-224. All lysates were equivalent to 100 µg total protein. As positive control, the CG kinetics of vector lysate containing 5 µg pCG was measured. The kinetics were measured as in (A).
(C) *Shigella* (M90T, $3x10^8$/ml) was incubated with the different lysates at a total protein concentration of 52 μg/ml. For the wt lysate this concentration corresponded to 0,01 NE activity units (as described in A). As negative control, vector lysate was used. V+pNE corresponding to 0,01 NE units served as positive control. Lysates of the NE-like mutants were also tested in the presence (+) of the NE inhibitor (NE-CMK, 200 µM). The reaction mixtures were incubated for 1 h at 37°C. After bacterial lysis, aliquots were analyzed by SDS-PAGE and subsequent immunoblotting using an anti-IcsA and OmpA antibody.
(D) *Shigella* (M90T, $3x10^8$/ml) was incubated with the 216-218 lysate at different concentrations of total protein. Undiluted lysate (1:1) corresponded to 52 μg/ml total protein. As negative controls, vector lysate (V) and wt lysate in the presence (+) of the NE inhibitor were used. As positive control, wt lysate in absence of the NE inhibitor was used. The experimental set-up is described in (C).

4 Discussion

An important question in protein biology is how proteases recognize their substrates. The super-family of serine proteases, for example, contains peptidases with very diverse functions such as digestion, degradation, blood clotting, cellular and humoral immunity, fibrinolysis, fertilization, embryonic development, protein processing and tissue remodeling (Rawlings and Barrett 1993). Additionally, the individual proteases have diverse functions. For instance, NE cleaves extracellular matrix proteins but it also has an important role in host defense. In neutrophils, NE is the key component in preventing the phagolysosomal escape of *Shigella*. NE specifically cleaves the *Shigella* virulence factors but it does not target proteins important for bacterial homeostasis or secreted proteins that are not associated with virulence. Thus, NE likely inhibits the interaction of *Shigella* with neutrophil cytoplasmic proteins. However, it is not known how NE recognizes its substrates. Moreover, it has remained unclear why other members of the chymotrypsin family of serine proteases do not target these virulence factors, although they are homologous to NE and are also in contact with the bacterial effectors *in vivo*. For example, CG, like NE, is part of the azurophilic granules of neutrophils. These two proteases are structurally almost identical, yet CG does not cleave *Shigella* virulence factors. In the present study, we approached the question of the specificity of NE for virulence factors from different angles. We first analyzed the substrate for a NE recognition motif. Secondly, we tried to identify the residues within NE that are crucial for the substrate specificity by a structure-function analysis of NE.

4.1 Do virulence factors contain a recognition motif for NE?

4.1.1 A recognition motif for NE was not detected in the primary sequence of IpaB

To test if NE recognizes a primary sequence motif in its substrate we analyzed twelve IpaB deletion mutants for their susceptibility to NE cleavage. These mutants were functional since they complemented a *Shigella ipaB* deletion strain in epithelial cell invasion and macrophage cytotoxicity when introduced on a plasmid (Guichon et al. 2001). We found that NE cleaved all these mutant proteins, albeit with slightly different patterns. For example, the IpaB mutant harboring a deletion of the amino acids 207-216 showed a cleavage pattern that suggests a higher susceptibility to NE compared to wildtype IpaB. In

contrast, an IpaB mutant harboring a deletion of the amino acids 217-226 seemed less susceptible to NE cleavage than wildtype IpaB. One explanation of these findings is that the relatively large amino acid deletions altered the folding of IpaB in a way that the accessibility of the NE cleavage sites was facilitated or reduced depending on the location of the deletion. However, this putative altered folding did not interfere with the function of the IpaB protein, since we restricted the analysis to testing functional IpaB mutants as mentioned above. By using only functional mutants we wanted to avoid scoring mis- or unfolded proteins. As a result, we were restricted to analyze a small part of the protein, covering only 21% of the 580 IpaB residues. It is therefore possible that a NE recognition motif in the primary amino acid sequence exists but was not identified using this approach. However, several reasons argue against the possibility of a primary sequence motif.

First, NE cleaves not only IpaB but also other virulence proteins, such as the Ipa proteins. IpaA and IpaC, and IcsA. Although these proteins are important for host cell manipulation, they belong to different protein families and hence are not homologous. Additionally, mass spectrometric analysis of NE derived cleavage products of IpaB, A, and C did not reveal a consensus sequence. Another argument against a primary sequence motif in the NE substrates results from a sequence comparison of the synthetic NE peptide substrate with the virulence factors. This peptide was used to measure NE activity. It consists of four amino acids (valine at the P1 position followed by proline and two alanines) linked to a chromophore. It was shown that NE cleaves peptide substrates with high efficiency if the substrates carry valine at the P1 and proline at the P2 position (Zimmerman and Ashe 1977; Marossy et al. 1980; McRae et al. 1980; Harper et al. 1984). A valine-proline sequence tag within the virulence factor could therefore serve as a NE recognition motif. However, we were unable to find such a tag in IpaB. Nevertheless, it might be worth to scan IpaB and other virulence factors for the occurrence of a “side chain profile” resembling the residues of the peptide substrate. Finally, one could assume that the number of residues after which NE can cleave is reduced in *Shigella* proteins not targeted by NE compared to virulence factors. NE prefers to cleave after valine but also after leucine or alanine (Powers et al. 1977; Nakajima et al. 1979; Marossy et al. 1980; Harper et al. 1984). However, the percentage of these residues does not significantly vary between the different protein groups. For example, alanine, valine and leucine constitute 17% of the primary sequence of IcsA, which is cleaved by NE, whereas they represent 26% in OmpA, which is not cleaved by NE.

4.1.2 The secondary and tertiary structures of the substrate do not affect the specificity of NE for virulence factors

The presence of discrete cleavage products of IpaB upon NE treatment suggests that IpaB was folded in the *Shigella* culture supernatant and that NE only attacked some of the potential cleavage sites. The question is if NE specifically recognizes these cleavage sites or if virulence factors in contrast to non-virulence factors display some of their valines on the surface of the folded protein. To address the latter question one would need to compare multiple structures of each protein group and search for exposed amino acid stretches containing valine. However, only few virulence factors have been crystallized to date, which would make this a challenging approach. Therefore, we tested if the secondary or higher order structures of IpaB are a prerequisite for cleavage by NE. In fact, NE cleaved denatured IpaB with the same cleavage pattern as native IpaB. Denatured IpaB was slightly more susceptible to NE than native IpaB as the 62 kDa band of IpaB was degraded to a greater extent than in native IpaB. This result indicates that secondary structures like coiled-coils do not serve as recognition motif for NE. Such structures have been proposed as common features for virulence factors (Pallen et al. 1997). In addition, this also suggests that the cleavage of IpaB by NE does not require higher order structures beyond the secondary structure level. However, we cannot exclude the possibility that denatured IpaB refolded during the 1-2 h incubation time at 37^0C in the NE cleavage experiment.

It is also possible that IpaB is generally not folded in the *Shigella* supernatant. Secreted effectors such as IpaB are released through a 2-3 nm wide pore within the needle-like structure of the type three secretion apparatus in *Shigella* (Blocker et al. 1999). Folded IpaB as globular protein likely possesses a diameter of approximately 6 nm, which suggests that IpaB passages the needle in an unfolded state. Since IpgC, the chaperone of IpaB, remains within the cytoplasm of *Shigella*, one could assume that IpaB stays unfolded in the supernatant.

IpaB is thought to form an extracellular complex with IpaC after secretion across the bacterial membrane and to facilitate further effector delivery (Blocker et al. 1999; Hayward and Koronakis 1999). One could assume that the binding to IpaC is a prerequisite for folding of IpaB. Since we used supernatant from *Shigella* carrying ipaB on a high-copy plasmid, one could argue that overexpression of IpaB could outnumber the IpaC molecules leading to an accumulation of mis- or unfolded IpaB. However, it was shown that the amount of IpaB secreted by this *Shigella* strain is not significantly increased in comparison

to the wildtype strain (Guichon et al. 2001). Therefore it is rather unlikely that the IpaB analyzed in this study was different from IpaB secreted by a wildtype *Shigella* strain.
Taken together, we were unable to detect a recognition motif for NE in the primary or higher order structures of the NE substrate IpaB. Since NE has to recognize and bind the virulence factors before cleavage, the role of the substrate in this interaction remains to be elucidated. One approach could be to N-terminally sequence NE derived cleavage products of IpaB and map these sequences to the primary structure of IpaB. Subsequent analysis of the biochemical character of the amino acids C- and N-terminal to the scissile bond could yield a common pattern, for example a stretch of hydrophobic residues. The sequences of *Shigella* virulence factors and other enterobacteriacae could then be examined for this pattern.

4.2 The specificity of NE for virulence factors is encoded in NE

Next, we addressed the question of localization of NE specificity by a functional analysis of wildtype and mutant NE. We hypothesized that a recognition motif for virulence proteins existed in NE and that it should be possible to mutate this motif without comprising the catalytic activity of the protease. More specifically, we speculated to identify a NE mutant that was still active towards its peptide substrate but would no longer recognize and cleave virulence factors like IpaB. For this purpose we expressed recombinant NE.

4.2.1 Recombinant expression of NE

In neutrophils, NE is synthesized as an inactive zymogen that requires the removal of amino acids at the N- and C-terminus for full activity. As a result, active mature NE starts with isoleucine instead of methionine. Since bacteria lack the proteases needed for the NE processing, we assumed that recombinant expression of the full-length protein in *E. coli* would not result in an active protein. Thus, we expressed recombinant mature NE that carried an additional methionine as first aminoterminal residue. However, this recombinant NE was not active, since it did not cleave the NE peptide substrate. Interestingly, it was mentioned that expression of the same mature NE in RBL-1 cells did also not result in an active protein (Li and Horwitz 2001). Since expression of the full-length protein in RBL-1 cells does yield active NE [our results; (Li and Horwitz 2001)], it is likely that the methionine with its bulky aromatic side chain interferes with the proteolytic function of the

protein. But it is also possible that the N-terminal two-step processing of NE is necessary for correct post-translational modification or folding of the enzyme. Another reason why *E.coli* derived NE was not active could be the inability of prokaryotes to glycosylate proteins. NE carries two asparagine-linked side chains, which might be important for the activity of the enzyme (Sinha et al. 1987; Watorek et al. 1993). However, according to the structure of NE these sugar chains are located at the surface of the protein away from the active site (Bode et al. 1989). Thus it is rather unlikely that they are involved in substrate recognition or important for enzymatic activity. Furthermore, the RBL-1 cells target NE to granules just as neutrophils do (Gullberg et al. 1994). This may support folding of NE and is likely to prevent auto-degradation of the enzyme as it is less active in the low pH environment of the granules and when attached to a granular matrix (Avila and Convit 1976). In summary, RBL-1 cells seem to provide optimal conditions to express a protease of the azurophilic granules of neutrophils.

4.2.2 The specificity of NE for virulence factors is encoded in NE

Since the analysis of the substrate could not answer why and how NE specifically recognizes virulence factors, we approached this question by mutational analysis of the NE protein. NE belongs to a large family of serine proteases with sequence and structural similarity but with very different biological functions. CG does not degrade *Shigella* virulence proteins, although it is as abundant in the azurophilic granules as NE and belongs to the same subfamily of chymotrypsin-like serine proteases. Additionally, CG and NE are not only homologous, but their crystal structures are almost identical. Yet, specific differences should explain why NE but not CG targets virulence factors. Substrate specificities among this group of serine proteases are considered to depend on amino acid variations in the substrate-binding cleft (Perona and Craik 1995). Therefore we analyzed the structure and amino acid composition of this cleft in NE and CG. We identified loci where single or multiple amino acids were strikingly different between the two enzymes. We assumed that replacement of these residues in NE by their structural CG counterparts or by the non-polar amino acid alanine would alter the NE specificity in a way that the NE mutants would not be able to target *Shigella* virulence proteins any more. All residues selected for mutation were either part of the NE substrate binding pockets or of surface loops containing residues of the different pockets. In general, these loops connect the walls of the pockets without necessarily contacting the substrate residues directly (Hedstrom et al.

1992). Since loop sequences are often unique in individual proteases, they have been suggested to play a role in determining substrate specificity (Hedstrom et al. 1992). The following chapters discuss the functional profiles of the generated NE mutants with regard to the location of the affected residues in the sequence and in the three-dimensional structure (see also chapter 3, figures 3.10 and 3.11 and for sequence alignment see appendix, table 5.1).

NE mutants 35-41 and 58A-61

The residue exchanges in the NE mutants LRGGHF 35-41 IQSPAGQSR and VANVNVR 58A-61 WGSNINV affected the S1-3' pockets and the corresponding surface loops [figure 4.1 and (Bode et al. 1989)]. However, these two NE mutants displayed different phenotypes.

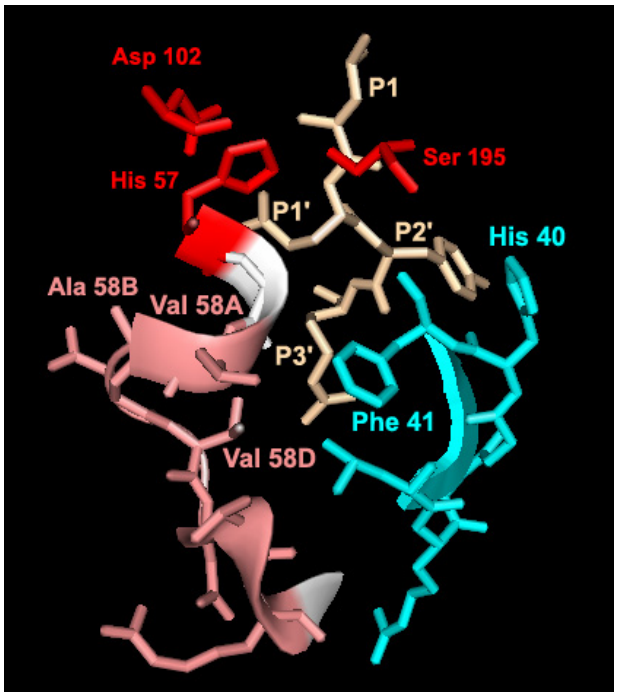


Fig 4.1: Schematic representation of the NE residues 35-41 and 58A-61.
The NE segments 35-41 (cyan) and 58A-61 (salmon) interact with the amino acids of substrate C-terminal to the scissile bond (P1'-P3' in wheat). The catalytic residues aspartate, histidine and serine are shown in red. The residues discussed in the text are labeled using the three-letter code for amino acids. The picture was generated using Pymol and residues are depicted as sticks and cartoon (DeLano 2002). The representation is based on the crystallization of NE with the inhibitor TOM (Bode et al. 1986b).

In the NE mutant 35-41 the phenylalanine (F) at position 41 was exchanged by an arginine (R). Interestingly, this exchange did not affect the ability of the mutant to cleave the NE peptide substrate and *Shigella* virulence factor, although this residue had been suggested to influence specificity (Bode et al. 1989). According to the structure of NE bound to an inhibitor, the phenylalanine interacts with the P1' and the P3' residue of this substrate [figure 4.1 and (Bode et al. 1986b)]. In the mutant 35-41, arginine is at this position and its side chain is positively charged in contrast to the one of phenylalanine. However, arginine in the context of CG is flexible, which might allow some adaptation to bound substrate residues. This could explain why the mutant was still able to cleave the NE substrates (Hof et al. 1996). In the rat mast cell protease II (RMCPII), another chymotrypsin-like serine protease, the residue segment 34-41 has been proposed to be important for substrate specificity (Perona and Craik 1995; Perona and Craik 1997). Yet, this suggestion was based on structural modeling but not on a functional analysis. In this study, we functionally show that the substrate specificity of NE is not encoded within this loop interacting with the P1'-P3' residues. It is therefore tempting to argue that protease residues interacting with the Pn' residues of the substrate are generally not important in determining NE specificity.

However, the mutant NE 58A-61 switched to a CG-like specificity for the peptide substrate and the exchanged residues do interact with the Pn' residues of the substrate [figure 4.1 and (Bode et al. 1986b; Bode et al. 1989)]. This indicates that a protease-substrate interaction C-terminal of the scissile bond actually is important for substrate recognition. The NE mutant 58A-61 had lost the specificity towards the NE but had acquired the specificity towards the CG peptide substrate. Additionally, the NE mutant 58A-61 did not target the *Shigella* virulence factors IpaB and IcsA. But since its CG activity was low, we cannot make any valid conclusions regarding the specificity towards *Shigella* virulence factors.

The switch in specificity for the peptide substrate could have been caused by the exchange of valine at position 58D. This valine in NE is known to interact with the substrate residue P3' [figure 4.1 and (Bode et al. 1989)]. However, the peptide substrate used in our assays is only composed of a chromophore C-terminal of the scissile bond and it is unclear if the NE valine contacts the chromophore. Therefore its role in NE specificity requires a more detailed analysis, for example, by generating a NE mutant that only lacks this valine.

As mentioned above, the CG activity of this mutant towards the CG peptide substrate was low as its CG kinetics was almost indistinguishable from the kinetics of endogenous proteases. It is possible that the introduced amino acids only partially conferred CG specificity and that we would need to exchange an elongated stretch of residues in this locus

to obtain a mutant with higher CG activity. Another explanation for the low CG activity could be that residues 58A and 58B are part of a β-sheet in NE and in close proximity to histidine 57, which is part of the catalytic triad [figure 4.1 and (Wei et al. 1988)]. This β-sheet might be disrupted by the exchange, which in turn could negatively influence the stability of the triad and thus the activity of the protein. Finally, the low CG activity might be caused by improper glycosylation. CG contains one potential N-glycosylation site (Watorek et al. 1993). This site is asparagine at position 60 and that is introduced into the mutant. It is possible that the mutant carries three asparagine-linked sugars, which interfere with the activity of protein.

Taken together, we showed that the segment 35-41 in NE is not important for NE specificity whereas the segment 58A-61 partly contributes to the specificity of NE.

NE mutants F192A, F192K and A213V

Most residues that we found to be significantly different in NE and CG were part of the substrate specificity pocket S1. The residues of this pocket have been considered as prime determinants for the specificities of the different chymotrypsin-like serine proteases, because they are complementary to the preferred P1 residue (Steitz and Shulman 1982; Berg JM 2003). Multiple residues including phenylalanine at position 192 define the S1 pocket of NE (figure 4.2). According to (Bode et al. 1989) the backbone of this phenylalanine contributes to the formation of the entrance of the S1 pocket and it is one the residues constricting the pocket towards the bottom. Importantly, the phenylalanine has been suggested to influence NE specificity (Bode et al. 1989). We assumed that exchange of this residue by its CG counterpart or by alanine would affect the specificity of NE. Surprisingly, both mutants, F192A and F192K, did cleave the NE peptide substrate as well as the *Shigella* virulence factors IpaB and IcsA. It is intriguing that replacement of phenylalanine by lysine (K) did not impact NE specificity, since lysine, in contrast to phenylalanine, is highly positively charged and not aromatic.

Interestingly, the NE mutant F192A was also able to cleave the CG peptide substrate, albeit with lower activity than the NE peptide substrate. The CG peptide substrate carries phenylalanine with its bulky and aromatic side chain at the P1 position. It is therefore possible that the introduction of alanine with its short side chain opened the entrance of the S1 pocket and thus allowed larger side chains to enter. Yet, it remains unclear how the introduced alanine elicited a CG activity. In summary, since the NE mutants F192A and

F192K retained NE specificity, the phenylalanine at position 192 does not seem to be a determinant for the specificity of NE, although it is part of the S1 pocket.

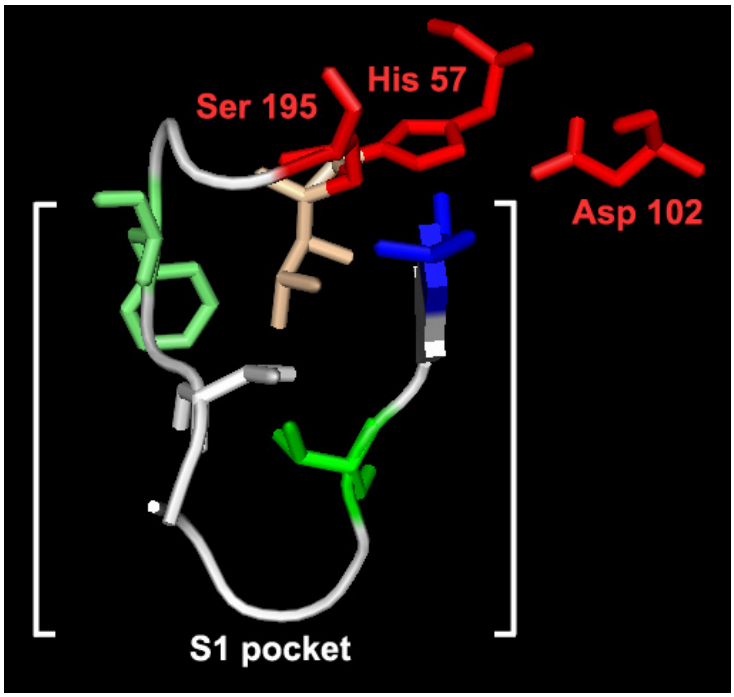


Fig. 4.2: Schematic representation of the S1 pocket of NE.
The residues phenylalanine at position 192 (lime) and alanine at position 213 (marine) form the entrance of the S1 pocket. The side chain of the substrates' P1 residue valine (wheat) points towards the pocket. The side chains of valine at position 190 (grey) and 216 (green) restrict the accessibility to the bottom of the pocket. The catalytic residues aspartate, histidine and serine are shown in red. The remaining NE residues are shown as cartoon in grey without side chains or are not shown at all. The picture was generated using Pymol (DeLano 2002) and is based on the crystallization of NE with the valine chloromethyl ketone inhibitor (Wei et al. 1988).

Alanine at position 213 in NE is another residue defining the S1 pocket (figure 4.2). It is thought to contribute to the constriction of the NE S1 pocket at its entrance and had been suggested to influence specificity of NE (Bode et al. 1986b; Bode et al. 1989). Interestingly, we found that exchange of the alanine by its CG counterpart valine did not influence the NE specificity. The NE mutant A213V specifically cleaved the NE peptide substrate as well as the *Shigella* virulence factors IpaB and IcsA. However, its NE activity was decreased in comparison to the NE activity of the mutants F192A and F192K. Since the isopropyl side chain of valine is larger than the methyl side chain of alanine, the presence of valine instead of alanine could have narrowed the entrance of the pocket and thus affected the NE activity of the mutant negatively. However, this amino acid exchange did not interfere with the specificity of the mutant for the biological substrates of NE. Interestingly, a topologically

close homolog of NE, the digestive chymotrypsin-like serine protease porcine pancreatic elastase (PPE), carries threonine at the position 213 and has been shown to preferably cleave after alanine (Powers et al. 1977; Zimmerman and Ashe 1977; Szabo et al. 1980). In contrast to valine, threonine is a polar residue but both side chains are branched in a similar way and thus present a similar steric restriction at the entrance of the S1 pocket. Therefore it is possible that the NE mutant A213V would also exhibit a higher activity towards an NE peptide substrate carrying an alanine instead of a valine at the P1 position.

Taken together, we were not able to change the specificity of NE by mutating single residues in NE at positions 192 and 213, although they are part of the NE S1 pocket. These observations are in accordance with the finding of (Graf et al. 1987), who analyzed the specificities of trypsin and chymotrypsin, two other prominent members of the chymotrypsin-like serine proteases. They showed that the specificity of trypsin could not be converted into a chymotrypsin-like specificity through exchange of a single S1 residue.

NE mutants F215Y and F215A

Phenylalanine at position 215 in NE is part of the S2 pocket and its side chain runs parallel to the side chain of the residue in the P4 position of the inhibitor [figure 4.3 and (Bode et al. 1989)]. This phenylalanine was either replaced by its CG counterpart tyrosine (Y) or by alanine (A). We found that the NE mutant F215Y specifically cleaved the NE peptide substrate and the *Shigella* virulence factors IpaB and IcsA. One explanation why the substitution of phenylalanine by tyrosine did not affect the character of NE is that the side chains of phenylalanine and tyrosine are identical except for a hydroxyl group at the aromatic ring of tyrosine. Therefore the steric and biochemical features of tyrosine resemble the ones of phenylalanine and maintain the bowl-shaped and rather hydrophobic character of the NE S2 pocket (Wei et al. 1988).

In contrast to tyrosine, the introduction of an alanine at position 215 did influence the activity and partially the specificity of the mutant enzyme towards the peptide substrate. F215A cleaved the NE peptide substrate but only with low activity since we could not measure the NE kinetics of the mutant. Furthermore, it acquired the ability to cleave the CG peptide substrate but this CG activity was also low. It is likely that alanine, whose side chain only consists of a methyl group, interfered with the character of the NE S2 pocket. This could have lead to an incorrect positioning of the P2 residue proline, which maybe influenced positioning of the P1 residue of the substrate or the binding of the peptide substrate in general. As mentioned above, the side chains of phenylalanine at position 215

and of the P4 amino acid of the substrate are in close contact. Therefore it is possible that the introduction of alanine at position 215 negatively affects the putative interaction of these side chains. Since proline and alanine are at the P2 and P4 positions in the NE and the CG peptide substrate, this might explain why both activities of the mutant NE were low. However, it does not explain why the NE mutant F215A was also able to cleave the CG peptide substrate. It is possible that the phenylalanine influences the S1 pocket in the native and soluble NE protein and therefore its exchange by alanine with its short side chain enlarged the S1 pocket allowing cleavage after a bulky residue. Another explanation could be that phenylalanine at position 215 influences the correct formation or function of the NE surface loop spanning the amino acids 163 to 181, because its side chain is in close proximity to the residues 177 and 180. This surface loop seemed important for substrate binding of NE in preliminary experiments (data not shown) and had been suggested to be crucial for specificity of trypsin (Perona and Craik 1997).

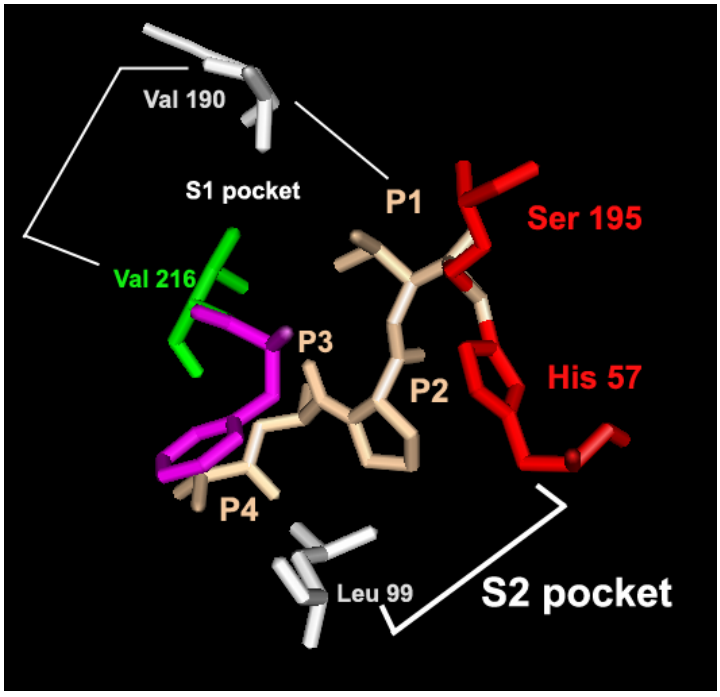


Fig. 4.3: Schematic representation of the S2 pocket of NE.
The S2 pocket of NE is formed among other residues by phenylalanine at position 215 (magenta), histidine (His 57) and leucine (Leu 99) (Bode et al. 1989). The residues of the substrate N-terminal to the scissile bond are shown in wheat (P1-P4). The side chain of phenylalanine at position 215 runs parallel to the substrate residue P4. Parts of the S1 pocket (Val 190 and Val 216) are shown for better orientation. The catalytic residues histidine and serine are depicted in red. All residues are presented as sticks The picture was generated using Pymol (DeLano 2002) and is based on the crystallization of NE with the valine chloromethyl ketone inhibitor (Wei et al. 1988).

Taken together, the substitution of phenylalanine at position 215 in NE with its CG counterpart tyrosine did not influence the NE specificity. In contrast, the substitution of that residue with alanine reduced the NE activity of the mutant and simultaneously allowed the cleavage of the CG peptide substrate. Since the NE as well as the CG activity of the NE mutant F215A was too low to measure kinetics, we are unable to draw any conclusions with regard to the activity against virulence factors.

NE mutants 216-218 and 216-224

The NE mutants VRGG 216-218 GKSS and VRGGCASGLY 216-224 GKSSGVP affected several NE specificity pockets as well as a surface loop, which is constituted by the residues 217-226 (figures 4.4 and 4.5). We could show that these two mutants lost the specificity for the NE peptide substrate but gained the ability to cleave the CG peptide substrate. Interestingly, the CG activity of the NE mutant 216-218 was approximately ten times higher than the CG activity of the NE mutant 216-224 per total protein concentration of the cell lysate. Since we did not purify the recombinant protein, it remains to be determined if this mutant actually presented a CG-like enzyme with a high substrate turnover rate or if the mutant protein was expressed to a higher level than NE mutant 216-224. Furthermore, NE mutant 216-224 did not target the *Shigella* virulence factors, whereas the NE mutant 216-218 showed marginal IpaB and IcsA degradation. Because of this residual NE specific activity, it is tempting to argue that the complete NE segment of amino acids 216-224 is crucial for the NE specificity for virulence factors. The segment 219-224 constitutes almost the complete surface loop that was suggested to influence specificity of chymotrypsin-like serine proteases (Perona and Craik 1997). Additionally, the residues 223 and 224 were also proposed to influence NE specificity (Bode et al. 1989). However, the NE mutant 216-218 did not cleave the *Shigella* virulence factors when its CG activity units were comparable to the ones of NE mutant 216-224. This strongly suggests that the four NE amino acids 216-218 are sufficient to encode for the NE specificity. It nevertheless might be interesting to generate a NE mutant in which only the residue segment 219-224 is exchanged and test its ability to cleave virulence proteins.

Assuming that the NE segment 216-218 does encode for the NE specificity, it is tempting to speculate that only one of the four residues determines this specificity. The two residues at position 217 and 218 in NE are glycines, which were replaced by two serines in the mutants. These residues possibly interact with the P5 position of the substrates [figure 4.4 and (Bode et al. 1989)]. In both peptide substrates this position is composed of a succinyl group but it

carries different modifications. The succinyl group in the CG peptide substrate contains a polar hydroxyl group whereas the methoxysuccinyl group in the NE peptide substrate harbors a non-polar methyl group at this position. It is possible that the introduced residues are able to interact with hydroxl- but not with methyl groups, which in turn influences specificity. Functional analysis of a NE mutant in which only the two glycines are replaced by serines should clarify the role of these residues in NE specificity. The residue 216B in NE is an arginine and its side chain points along the side chain of the P4 residue of the substrate [figure 4.4 and (Bode et al. 1986b; Bode et al. 1989)]. Since arginine was replaced by lysine that has similar biochemical features, it is rather unlikely that this exchange conferred the switch of specificity in the NE mutant 216-218. The most likely candidate for determining specificity is the residue at position 216. It had been speculated earlier that this residue might be important for specificity of trypsin (Perona and Craik 1997).

In both NE mutants 216-218 and 216-224, valine at position 216 was substituted by its CG counterpart glycine. The side chains of both valine and glycine at this position reach into the S1 pockets of NE and CG, respectively. Thereby the residues define the size of the S1 pockets (figure 4.2 and see also chapter 1, figure 1.7). The side chain of glycine, which only consists of hydrogen, is smaller than the one of valine, resulting in an enlarged S1 pocket. Thereby it allows the bulky and aromatic side chain of phenylalanine, which is the preferred P1 residue in CG peptide substrates, to enter to the base of the pocket. In contrast, NE does not cleave peptide substrates with phenylalanine at the P1 position (Harper et al. 1984), possibly because the valine sterically hinders the access of large side chains. In addition, the side chain of valine also contributes to the hydrophobic character of the NE S1 pocket by burying the acidic aspartate at position 226 (Navia et al. 1989). Therefore, the replacement of valine by glycine could not only affect the geometrical but also the biochemical character of the S1 pocket in the mutant. Interestingly, trypsin also contains a glycine at position 216. It cleaves peptide bonds after arginine or lysine, whose side chains are the longest among the twenty common amino acids. Replacement of this glycine with alanine resulted in an almost complete loss of trypsin activity when measured using peptide substrates (Hedstrom et al. 1992). Since the size of alanines' side chain is in between the one of glycine and valine, it is possible that this trypsin mutant would be able to cleave the NE peptide substrate. Furthermore, it would be interesting to test if replacement of this glycine with valine would confer NE specificity to trypsin. However, it was stated that introduction of residues of the NE S1 site into trypsin failed to confer specificity towards elastase-specific substrates (Perona et al. 1995). Eventually, the generation and analysis of a NE mutant in

which only the valine at position 216 is replaced by glycine could prove if the specificity of NE for virulence factors is encoded in this residue. If this was the case, it would mean that the character of the S1 pocket determines substrate specificity. Consequently, it would indicate that in virulence factors, in contrast to non-virulence proteins, valines and other residues after which NE cleaves are more accessible than residues after which CG cleaves.

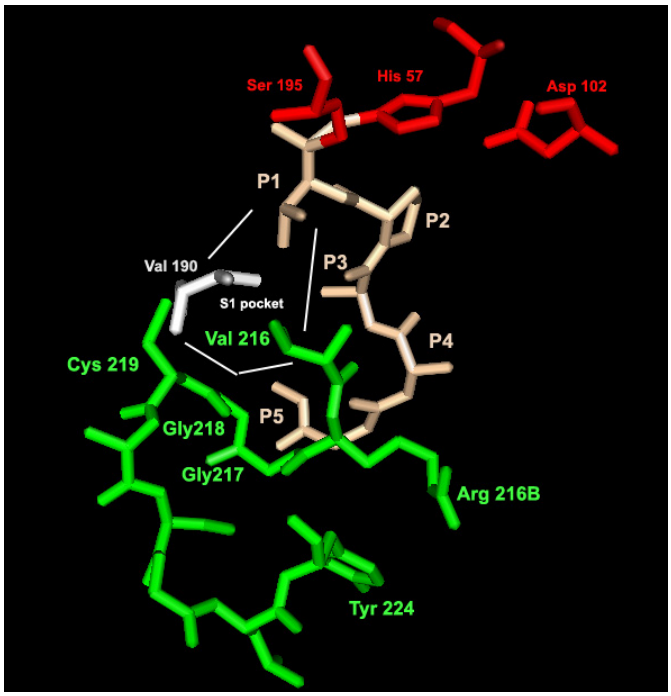


Fig.4.4: Schematic representation of the NE segment 216-224.
The residues 216-224 (green) are part of a surface loop and interact with the residues of the inhibitor N-terminal to the scissile bond (P1-P5 shown in wheat). In addition to Val 216 parts of the S1 pocket (Val 190) are shown for better orientation. The catalytic residues aspartate, histidine and serine are depicted in red. All amino acids are presented as sticks. The representation was generated using Pymol (DeLano 2002) is based on the crystallization of NE with the valine chloromethyl ketone inhibitor (Wei et al. 1988)

Taken together, we demonstrated that the residue segment 216-224 is crucial for the NE specificity for virulence factors. Additionally, by inserting the analogous CG residues we were able to introduce CG specificity for peptide substrates. Thereby we functionally proved the hypothesized importance of the surface loop 217-225 in substrate specificity among chymotrypsin-like serine proteases (Perona and Craik 1997).

NE mutant N98L and N98A

Asparagine at position 98 in NE is part of a protruding loop segment that in CG comprises the residues 94-99 (Hof et al. 1996). This residue was replaced by its structural counterpart leucine or by alanine, resulting in the two NE mutants N98A and N98L. Interestingly, the substitution of asparagine by leucine did not influence the NE specificity for the peptide substrate or the *Shigella* virulence factors. Leucine carries a nonpolar side chain, whereas the side chain of asparagine is polar. However, both side chains are branched in a similar way, which might explain why the exchange did not influence the NE specificity of the mutant. In contrast, replacement of asparagine with alanine led to a complete change of specificity. The NE mutant N98A did not cleave the NE peptide substrate and *Shigella* virulence factors but cleaved the CG peptide substrate. As mentioned above, the asparagine is part of a surface loop, and this loop was proposed via structural computing to influence specificity of an enteropeptidase of the chymotrypsin-like family (Perona and Craik 1995). However, the asparagine was not suggested to influence specificity of NE. Furthermore, alanine is a nonpolar residue like leucine and the asparagine in NE does not directly contact the inhibitor (figure 4.5). It is therefore difficult to explain why the introduction of alanine had such a strong effect. Since the side chains of alanine and leucine differ in size, it is possible that the side chains' steric character at this position is important for the interaction with the substrate N-terminal to the scissile bond and thus for the specificity of the enzyme. To understand the critical function of this NE residue at position 98 in substrate recognition, one would have to analyze the crystal structure of this mutant bound to IpaB.

Taken together, we proved asparagine at position 98 to be a determinant of NE specificity. The replacement of this single amino acid with alanine introduced CG specificity to the mutant enzyme. This is in contrast to the hypothesis of (Krem et al. 1999). They postulate that the C-terminal part, starting at residue 189, encodes for the function of serine proteases. Additionally, it is not possible to confer chymotrypsin specificity into trypsin by replacement of a single residue. To achieve this transfer in specificity, one residue of the S1 pocket and two adjacent surface loops have to be exchanged (Hedstrom et al. 1992).

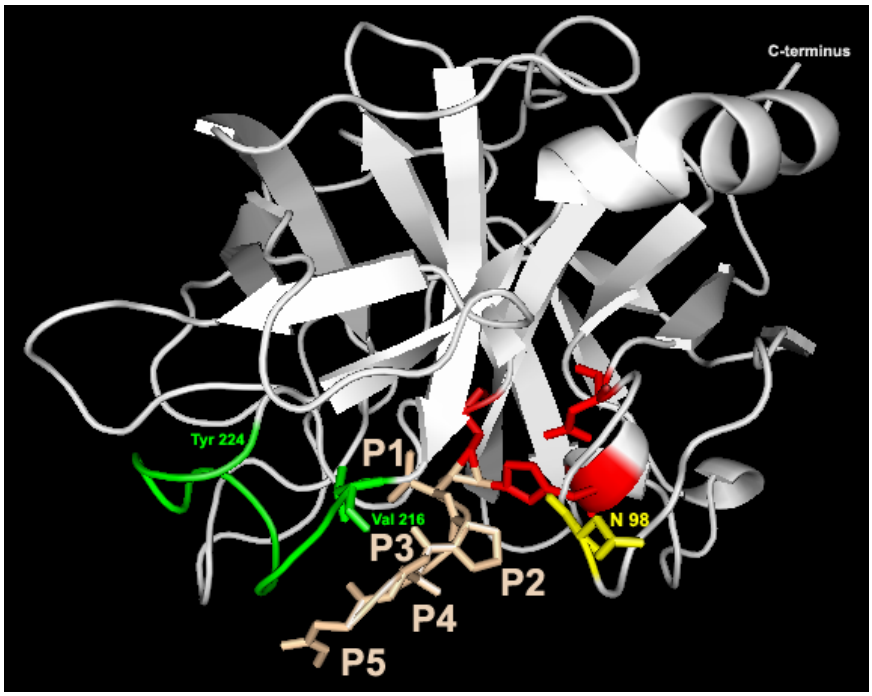


Fig. 4.5: Schematic representation of NE.
The residues 217-225 form a surface loop (green). The valine at position 216 (green) is shown with its side chain. The asparagine at position 98 (yellow) does not directly contact the inhibitor (P1-P5, wheat) and it is located on the surface of the protein. The catalytic residues aspartate, histidine and serine are shown in red. The remaining NE residues are shown as cartoon in grey. The two barrels of β-sheets (arrows) and a single α-helix are shown. The presentation was generated using Pymol (DeLano 2002) is based on the crystallization of NE with the valine chloromethyl ketone inhibitor (Wei et al. 1988).

Conclusion

In the present study we identified the residues at position 98 and 216-224 to be critical for NE specificity. Mutation in these residues to alanine or the analogous CG residues changed the specificity CG-like. Furthermore, the NE mutants N98A, 216-218 and 216-224, as CG, did not degrade *Shigella* virulence factors, although they were able to cleave the CG peptide substrate. This finding contributes to our understanding of how proteases recognize their substrates, since it shows that single residues apart from the catalytic center can determine the interaction of the full-length enzyme with its substrate. We assume that introduction of these NE residues into CG would confer NE specificity to the CG mutants. To further underline the biological importance of our results, it would be very interesting to perform *in* and *ex vivo* experiments. It was shown that NE null mice are more susceptible to Gram-negative bacteria, whereas CG knockout mice are susceptible to infections with Gram-positive bacteria (Tkalcevic et al. 2000; Reeves et al. 2002). Therefore, mice expressing mutant instead of wildtype NE should also be more susceptible to infections with Gram-negative bacteria. Furthermore, isolated neutrophils from these mice should allow the

escape of *Shigella* from the phagolysosome to the cytoplasm, as it was observed in neutrophils from NE null mice (Weinrauch et al. 2002). Additionally, wildtype and transgenic mice expressing our identified NE mutants could be infected with various pathogens and this way the spectrum of NE specificity *in vivo* could be assessed. On the other hand, one could test if the three CG-like NE mutants do not only cleave the CG peptide substrate but are also able to target biological substrates of CG such as the human brahma protein (Biggs et al. 2001).

Our results also present an important finding with regard to the evolution of proteases. The members of the large family of chymotrypsin-like serine proteases derived from a common ancestor and exist in pro- and eukaryotes. This parental enzyme is thought to possess a prototypical fold containing the catalytic machinery. However, the broad divergence in substrate specificity within this protease family is incompletely understood (Krem et al. 1999). The variety in function is thought to have derived from mutation of residues that are not involved in the catalytic or structural function. Furthermore, it has been postulated that enzymes of this family that evolved early are less diverse and thus less stringent in their substrate P1 preference. In contrast, proteases that evolved late, such as NE and CG, are very stringent in their P1 substrate specificity leading to specific functions and thus individual ecological niches of the proteases (Perona and Craik 1997). We show that these niches can be switched by mutation of a single residue that is not involved in the architecture of the S1 pocket. The interaction of the immune system and pathogens is characterized by a constant battle of detection and elimination on the one and evasion on the other side. Pathogens usually have a short life span, which facilitates the appearance of new variants that can be selected to escape the host immune system. The fact, that the specificity of NE can be converted to that of CG by a single mutation, even though their sequence identity is only 37%, could indicate that evolution lead to specific chymotrypsin-like serine proteases but that the ability to exchange functions was retained in this group of proteases. Thereby, the chances are increased that a new pathogen with novel virulence factors will encounter a mutant protease that can recognize and degrade the pathogenic factors.

In summary, this study has defined regions in NE distinct from the catalytic site that are important for recognition of virulence factors. In future studies, using crystal structures, X-ray scattering and High-throughput screening for substrates that are recognized by the mutant protein or peptide, we can begin to analyze whether these regions are important for binding to specific substrates and if conformational changes as a result of binding are important for enzyme specificity. These studies offer a potentially valuable tool to expedite

biochemical studies and drug design. Since NE leads to aberrant lung epithelium destruction and mucous production in cystic fibrosis, for example (Bruce et al. 1985; Birrer et al. 1994; Schuster et al. 1995) the results of this study could be useful for the design of a novel and precise NE inhibitor. On the other hand, the determination of the NE residues that are crucial for recognition of virulence factors could present a template for the design of new types of antimicrobials that block a bacterial infection by masking bacterial virulence factors.

5 Appendix

5.1 Expression of recombinant wt NE in bacteria

To express and purify recombinant NE we initially used the *E.coli* Rosetta™(DE3)pLys strain. It is a derivative of the BL21 *E. coli* strain that is widely used for protein expression and is deficient in the Lon and OmpT proteases (Phillips et al. 1984; Studier et al. 1990). Furthermore, these commercially available bacteria carry the T7 RNA polymerase gene under the control of an IPTG inducible promoter. To avoid basal expression of the polymerase and thus unspecific expression of NE, the bacteria contained the pLys plasmid encoding for T7 lysoszyme, a natural inhibitor of T7 RNA polymerase (Studier 1991; Zhang and Studier 1997). To enhance the expression of eukaryotic proteins, these bacteria contain tRNAs for the translation of codons rarely used in *E. coli* but that are present in the NE gene.

In neutrophils, NE is synthesized as an inactive zymogen that requires the removal of amino acids at the N- and C-terminus for full activity. As a result, active mature NE starts with isoleucine instead of methionine. Since bacteria lack the proteases needed for the NE processing, we assumed that recombinant expression of the full-length protein in *E. coli* would not result in an active protein. Therefore, we expressed recombinant mature NE that carried an additional methionine as first aminoterminal residue. In order to purify the recombinant protein we added a histidine tag to C-terminus of the protein.

The cDNA encoding for human mature NE was ligated into the expression vector pET-28(a)+ under the control of a T7 promoter. *E.coli* Rosetta™(DE3)pLys were transformed with the pET28(a)+/NE mature construct and expression of recombinant NE was induced by addition of IPTG for various times at 30 or 37°C. Bacterial lysates were tested for the presence of the recombinant protein by immunoblot analysis using an antibody against histidine. However, expression of recombinant NE was not detected (data not shown).

A reason why the protein was not expressed could be based on the DNA composition of the NE gene. The 5' sequence of this gene is very GC-rich (72%). The high GC content could lead to hairpin formation of the mRNA molecule or present an obstacle to the migration of the RNA-polymerase. In both cases protein translation would be prevented. Therefore five basepairs within the 5'-end of the DNA sequence encoding for the mature protein were silently exchanged to break GC stretches. The choice which basepairs should be mutated

was based on the PROTEOEXPERT program from Roche [www.proteoexpert.com (Roche-Applied-Science)], which is able to suggest basepair exchanges without altering the amino acid composition.

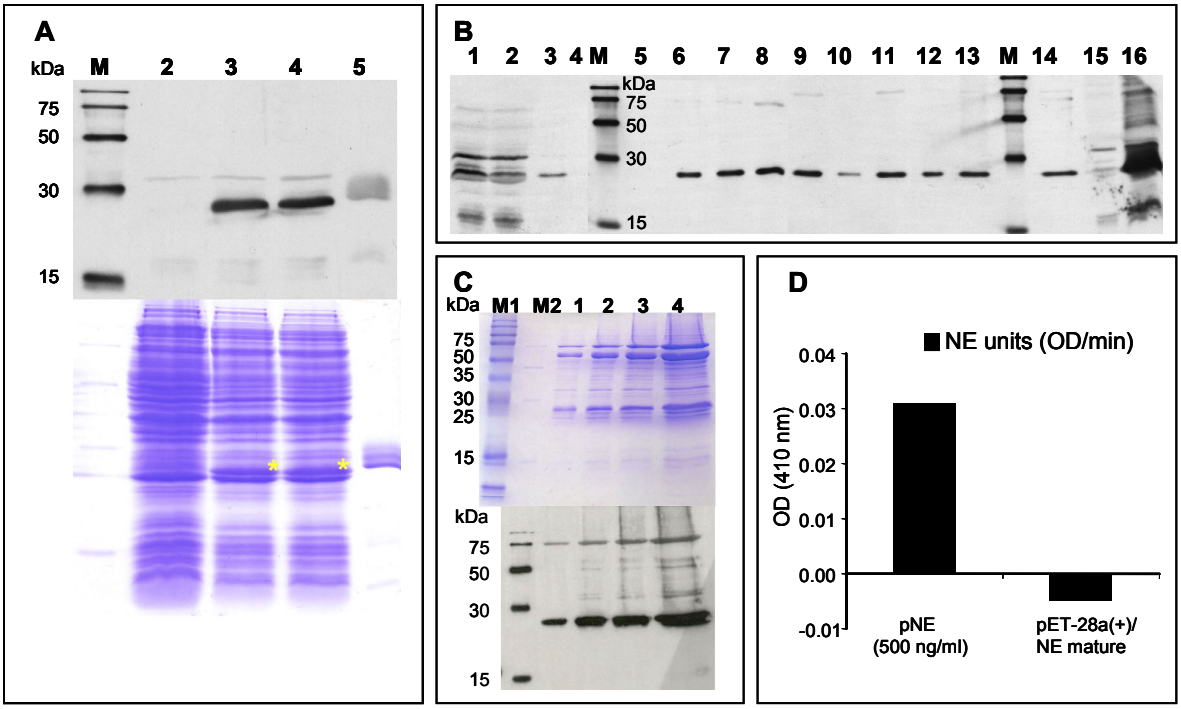


Fig. 5.1: Mature NE expressed in bacteria is not active.
(A) Expression of mature NE (C-terminal histidine tagged) after induction with IPTG for 4h at 37°C. Aliquots of bacterial lysates were analyzed by SDS-PAGE and subsequent Coomassie staining or immunoblotting using an anti-histidine antibody. In the Coomasie stained gel the expressed protein is marked with an asterisk. As negative control, *E.coli* were transformed with the empty expression vector and also treated with IPTG.
Lanes M: histidine ladder, 2: Rosetta+pET-28+(a), 3 and 4: Rosetta+pET-28+(a)/NE mature C-His, 5: 1 µg pNE
B. Purification of NE mature using a nickel column.
Aliquots of each purification step were analyzed by SDS-PAGE and subsequent immunoblotting using an anti-histidine antibody.
Lanes 1 and 2: flow through fractions, 3 and 4: washing fractions, 5 to 14: elution fractions, 15: Rosetta+pET-28+(a)/NE mature under non-inducing conditions; 16) starting material - Rosetta+pET-28+(a)/NE mature under inducing conditions.
C. Reconstituted recombinant NE mature analyzed by SDS-PAGE and subsequent Coomassie staining or immunoblotting using an anti-histidine antibody.
Lanes M1: size ladder, M2: histidine ladder, 1 to 4: 10, 20, 50 and 75 µl of the reconstituted eluate.
D. NE activity units of 250 µl reconstituted eluate and 500 ng/ml pNE. The eluate and pNE were added to the assay buffer containing the NE peptide substrate. The kinetics were measured by recording the OD at 410 nm wave-length every 30 sec over 3 min. The NE units represent the change in OD/min.

This altered DNA was ligated into pET28(a)+ leading to a C-terminal histidine tag. *E.coli* Rosetta™(DE3)pLys were transformed with this construct and expression was induced. We observed that the introduced basepair exchanges lead to an expression of mature NE (figure 5.1a). In order to test the activity of the enzyme, we purified the recombinant NE using a nickel column (figure 5.1b). After dialysis, lyophilization and reconstitution of the eluted protein (figure 5.1c), its enzymatic activity was tested with the NE peptide substrate. However, we were unable to detect enzymatic activity of the recombinant mature NE protein when compared to that of purified NE (figure 5.1d).

We concluded that expression of active recombinant NE was not possible in bacteria. In a next step, we tried to express mature NE in cell-free systems (Rapid Translation System *E.coli* from Roche Applied Science and TNT® Quick Coupled Transcription/Translation System from Promega) or in yeast. However, using these approaches we were also not able to detect expression of recombinant NE (data not shown).

5.2 Analysis of single-cell derived cell lines of the different NE mutants

As mentioned in chapter 3.2.5, RBL-1 cells were stably transfected with the eleven different pcDNA3/NE mutant constructs. As for recombinant wildtype NE, several single-cell derived cell lines of each mutant were tested for their NE activity. After retesting three cell lines with high NE activity, the cell line whose lysate showed the highest NE activity per cell number was selected for subsequent analysis (figure 5.2). Since no cell line of the four NE mutants 58A-61, N98A, 216-218 and 216-224 cleaved the NE peptide substrate, several of these single-cell derived cell lines were tested for their ability to cleave the CG peptide substrate (figure 5.3). Again, the cell line whose lysate showed the highest CG activity per cell number was selected for subsequent analysis.

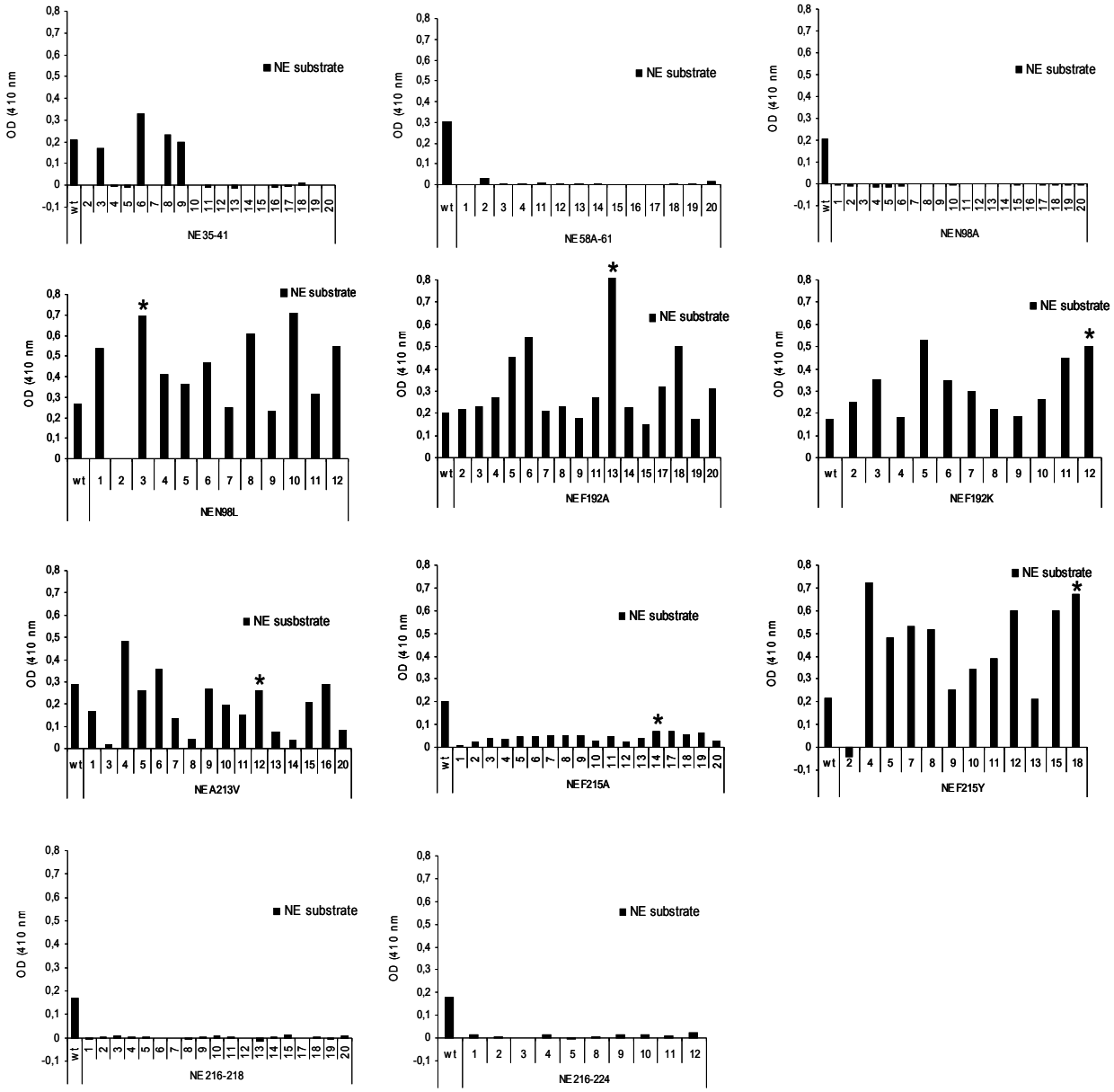


Fig. 5.2: NE activity of different cell lines of the 11 NE mutants.
NE activity of several single-cell derived cell lines of cells that carried the different pcDNA3/NE mutant constructs. Lysates were mixed with the NE peptide substrate and incubated for 30 min. The absorbance was read at 410 nm wave-length. The samples were normalized against a vector lysate that had been incubated with the substrate. As positive control, the substrate was added to wt lysate. All lysates were equivalent to $1x10^5$ cells and were treated equally. The clones marked with an asterisk were chosen for further experiments.

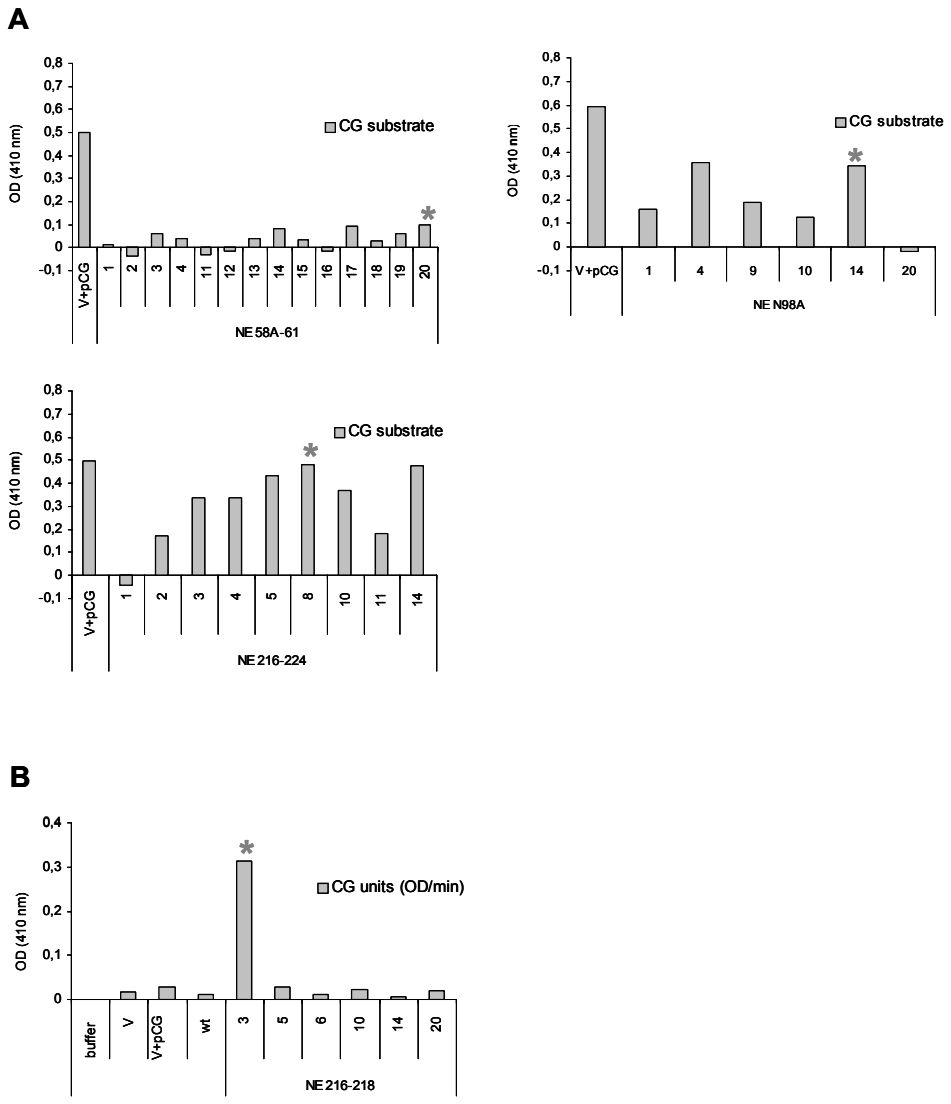


Fig. 5.3: CG activity of NE mutants 58A-61, N98A, 216-218, and 216-224.
(A) CG activity of several single-cell derived cell lines of cells individually transfected with pcDNA3/NE 58A-61, N98A, F215A or 216-224. Lysates were mixed with the CG peptide substrate and incubated for 30 min. The OD was read at 410 nm wave-length. The samples were normalized against a vector lysate that had been incubated with the substrate. As positive control, 1 μg pCG was added to vector lysate before the substrate (V+pCG). All lysates were equivalent to $1x10^5$ and treated equally. The clones with the highest activity were chosen for further experiments.
(B) CG kinetics of five single-cell derived cell lines of cells transfected with pcDNA3/NE 216-218. Lysates of $2,5x10^5$ cell equivalence/ml were assayed for cleavage of the CG peptide substrate over 3min. The absorbance at 410 nm was read every 30 sec. As negative control the kinetics of V lysate was measured. $2,5x10^5$ cell equivalence/ml V lysate spiked with 1,25 μg pCG was used as positive control. The CG units resembled the change in absorbance/minute.

5.3 Sequence alignment of NE, CG, trypsin and chymotrypsin

Table 5.1: Sequence alignment.

```
                                                    16                                  35
NE              MTLGRRLACL FLACVLPALL LGGTALASEI VGGRRARPHA WPFMVSLQLR
CG              ----MQPLLL L----LAFLL PTGAEA-GEI IGGRESRPHS RPYMAYLQIQ
Trypsin         ----MHPLLI LAFVGAAVAF PSDDD--DKI VGGYTCAENS VPYQVSLN--
Chymotrypsin    ---------- ---CGVPAIQ PVLSGL-SRI VNGEEAVPGS WPWQVSLQDK

                      41                    57        61
NE              ---GGHFCGA TLIAPNFVMS AAHCVANVNV RAVRVVL-GA HNLSRREPTR
CG              SPAGQSRCGG FLVREDFVLT AAHC----WG SNINVTL-GA HNIQRRENTQ
Trypsin         --AGYHFCGG SLINDQWVVS AAHC----YQ YHIQVRL-GE YNIDVLEGGE
Thymotrypsin    --TGFHFCGG SLINENWVVT AAHC----GV TTSDVVVAGE FDQGSSSEKI

                                98   102
NE              QVFAVQRIFE NG-YDPVNLL NDIVILQLNG SATINANVQV AQLPAQGRRL
CG              QHITARRAIR HPQYNQRTIQ NDIMLLQLSR RVRRNRNVNP VALPRAQEGL
Trypsin         QFIDASKIIR HPKYSSWTLD NDILLIKLST PAVINARVST LLLPSACA--
Chymotrypsin    QKLKIAKVFK NSKYNSLTIN NDITLLKLST AASFSQTVSA VCLPSASDDF

                          141
NE              GNGVQCLAMG WGL-LGRNRG IASVLQELNV TVVT------ ---SLCRRSN
CG              RPGTLCTVAG WG--RVSMRR GTDTLREVQL RVQRDRQCLR IFGSYDPRRQ
Trypsin         SAGTECLISG WGNTLSSGVN YPDLLQCLVA PLLSHADCEA SYPGQITNNM
Chymotrypsin    AAGTTCVTTG WGLTRYTNAN TPDRLQQASL PLLSNTNCKK YWGTKIKDAM

                             192 195                 213 216       224
NE              VCTLVRGRQA GVCFGDSGSP LVCNG----L IHGIASFVRG GCASGLYPDA
CG              ICVGDRRERK AAFKGDSGGP LLCNN----V AHGIVSY--- GKSSGVPPEV
Trypsin         ICAGFLEGGK DSCQGDSGGP VACNG----Q LQGIVSW-GY GCAQKGKPGV
Chymotrypsin    ICAG--ASGV SSCMGDSGGP LVCKKNGAWT LVGIVSW-GS STCSTSTPGV

NE              FAPVAQFVNW IDSIIQRSED NPCPHPRDPD PASRTH
CG              FTRVSSFLPW IRTTMRSF-- ------KLLD QMETPL
Trypsin         YTKVCNYVDW IQETIAANS- ---------- ------
Chymotrypsin    YARVTALVNW VQQTLAAN-- ---------- ------
```

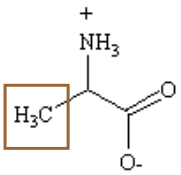
The sequences of human NE (Sinha et al. 1987), human CG (Salvesen et al. 1987), bovine trypsin (Le Huerou et al. 1990) and bovine chymotrypsin (Pjura et al. 2000) were aligned using the software MUSCLE (Edgar 2004). The numbering is according to the chymotrypsin numbering (Hartley B.S. 1971). The residues of the catalytic triad are shown in red and the amino acids that were mutated in NE are highlighted in the respective colors (see chapter 3, figure 3.11. The mature proteins start with isoleucine at position 16. The table was generated using CHROMA (Goodstadt and Pontig 2001).

5.4 Amino acids – abbreviations and structural formula

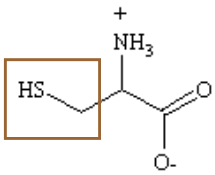
Amino acids can be grouped according to the chemical character of the respective side chain into non-polar, uncharged polar, acidic or basic residues. Tables 5.2-5.5 show the members of each group. The full name of the amino acids as well as the one- and three-letter abbreviation code is shown. The side chains are highlighted.

Table 5.2: Amino acids with non-polar side chains.

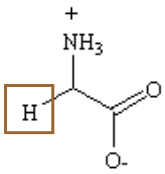
Alanine
(Ala or A)



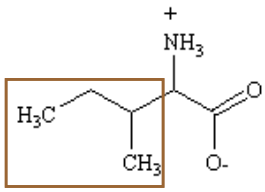
Cysteine
(Gly or G)



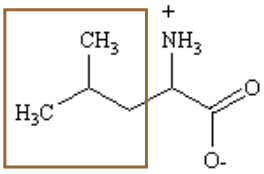
Glycine
(Gly or G)



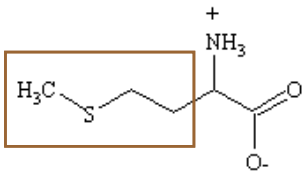
Isoleucine
(Ile or I)



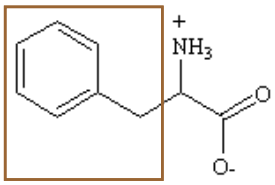
Leucine
(Leu or L)



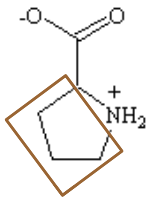
Methionine
(Met or M)



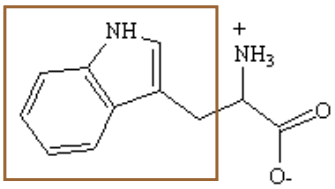
Phenylalanine
(Phe or P)



Proline
(Pro or P)



Tryptophan
(Trp or W)



Valine
(Val or V)

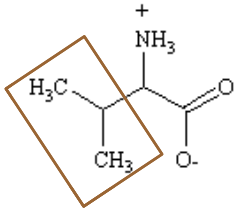
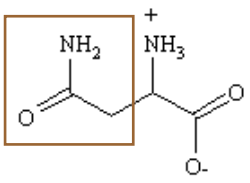
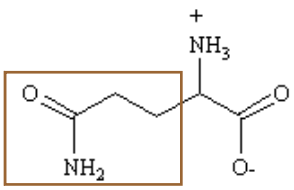


Table 5.3: Amino acids with uncharged -polar side chains.

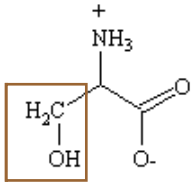
Asparagine
(Asn or N)



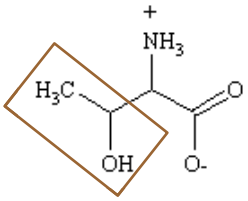
Glutamine
(Gln or Q)



Serine
(Ser or S)



Threonine
(Thr orT)



Tyrosine
(Tyr or Y)

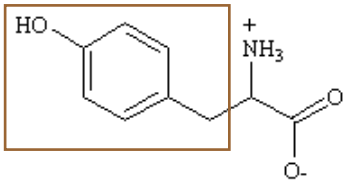
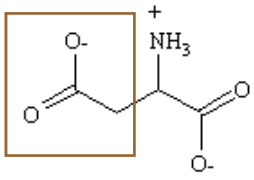


Table 5.4: Amino acids with acidic side chains.

Aspartic acid
(Asp or D)



Glutamic acid
(Glu or E)

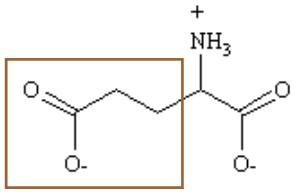
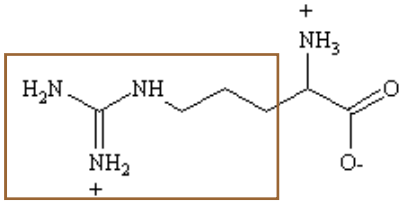
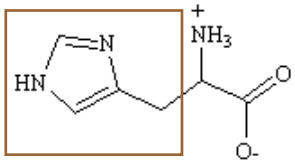


Table 5.5: Amino acids with basic side chains.

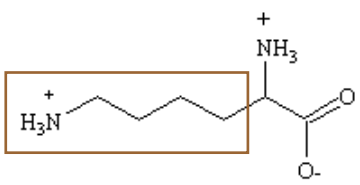
Arginine
(Arg or R)



Histidine
(His or H)



Lysine
(Lys or K)



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7 Abbreviations

The abbreviations for the individual amino acids are listed in chapter 5.4.

aa	amino acid
216-219	NE mutant VRGG216-219GKSS
216-224	NE mutant VRGGGSLY216-224GKSSGVP
35-41	NE mutant LRRGHF35-41IQSPAGQSR
59-61	NE mutant VANVNVR59-61WGSNINV
BPI	bactericidal permeability increasing protein
CAMP	cationic antimicrobial peptides
CG	cathepsin G
DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen
F192A	NE mutant F192A
F192K	NE mutant F192K
F215A	NE mutant F215A
F215Y	NE mutant F215Y
FCS	foetal calf serum
fMLP	aminoterminal formylated methionin bacterial peptide
HBSS	Hank's balanced salt solution
HEPES	N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)
hNGE	human neutrophil granule extract
IC	inhibitor cocktail
IcsA	intracellular spreading A
IL-8	interleukin 8
Il-18	interleukin 18
IL-1β	interleukin 1β
IpaB	invasion plasmid antigen B
LPS	lipopolysaccharides
MALDI	matrix assisted laser desorption ionisation
MeO	methoxy-
min	minutes
MS	mass spectrometry
N98A	NE mutant -N98A
N98L	NE mutant N98L
NE	neutrophil elastase

NE-CMK	NE inhibitor; MeO-Suc-AAPV-chloromethyl ketone
NETs	neutrophil extracellular traps
nm	nanometer
nt	non-transfected
OmpA	outer-membrane protein A
PAMPs	pathogen associated molecular patterns
PBS	phosphate-buffered saline
pCG	commercially available purified human CG
pI	isoelectric point
PMA	phorbol 12-myristate 13-acetate PMA
PMSF	phenylmethylsulphonylfluoride
pNE	commercially available purified human NE
PRRs	pathogen recognition receptors
RMSD	root mean square standard deviation
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	room temperature
RZPD	German Resource Center for Genome Research
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
sec	seconds
Suc	succinyl-
TCA	trichloroacetic acid
TLR	toll like receptors
TNF-α	tumor necrosis factor α
TOF	time of fligt
V213A	NE mutant V213A
V	vector
wt	recombinant wildtype NE
Z-GLF-CMK	CG inhibitor; benzyloxycarbonyl –GLF-chloromethyl ketone

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Poster abstracts

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Petra Averhoff, Hans-Markus Wenzel, Andrea Vortkamp
Role of Ext1 in Bone Development
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Selbständigkeitserklärung

Hiermit erkläre ich, daß ich die vorliegende Arbeit selbständig verfaßt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Ich versichere, daß diese Arbeit in dieser oder anderer Form noch keiner anderen Prüfungsbehörde vorgelegt wurde.
Der Inhalt der Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät I der Humboldt Universität zu Berlin vom 19.06.2002 ist mir bekannt.

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