FUNCTIONAL ANALYSIS OF THE P33 AND P55 DOMAINS OF THE

HELICOBACTER PYLORI VACUOLATING CYTOTOXIN

By

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To my best friend, best companion, and beloved wife Carmen.

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Before I came to Vanderbilt, I was blessed with the support, help, and love of my family, in particular, my mother Sonia and my wife Carmen. Without them I will not be here writing this thesis. My mother has been my inspiration, the key model for my working ethics and appreciation of life. My wife has been the driving force of my life for the past eight years. She is the reason why I chose to become a scientist and why we are here at Vanderbilt University today.

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BrdU	5-bromo-2-deoxyuridine
CBA	Cytometric bead array
CFSE	Carboxy fluorescein diacetate succinimide ester
FCS	Fetal calf serum
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescence protein
GTP	guanosine 5'-triphosphate
h	hour
His	Histidine
HIV	Human immunodeficiency virus
IB	Immunoblot
IF	Immunofluorescence
IPTG	Isopropyl-ß-D-thiogalactopyranoside
JAK	Janus kinase
kDa	Kilodaltons
LB	Luria Bertani broth
Leu	Leucine
LLO	Listeriolysin
MAPK	mitogen-activated protein kinase
MEM	Modified Eagle's medium
MHC	Major histocompatibility complex

NFAT	Nuclear factor of activated T cells
pAD	Plasmids encoding the GAL4 transcription-activation domain
PAI	Pathogenicity island
pBD	Plasmids encoding the GAL4 DNA-binding domain
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polimerase chain reaction
PE	Phycoerythrin
PI	Propidium iodide
PMA	Phorbol myristate acetate
RPTP	receptor protein tyrosine phosphatases
SD	Synthetic defined minimal medium
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SHP-2	Phosphatase SRC homology 2-phosphatase
STAT	Signal transducers and activators of transcription
TB	Terrific broth
TBS	Tris-buffered saline
TCR	T cell receptor
T_h	T helper
Trp	Tryptophan
YPAD	Yeast extract, peptone, dextrose

CHAPTER I

INTRODUCTION

Helicobacter pylori

Helicobacter pylori is a microaerophilic, spiral-shaped, Gram-negative bacterium that colonizes the human stomach (25, 87, 104). *H. pylori* resides primarily within the gastric mucus layer, but it can also adhere to gastric epithelial cells (5, 26, 82). More than 50% of the human population is thought to be infected with *H. pylori* (25, 87, 104). Once infection is established, *H. pylori* has no significant bacterial competitors and it can persist for decades in the absence of antimicrobial treatment. Infection with this bacterium induces extensive inflammation of the gastric mucosa (i.e. gastritis) and is considered to be a major risk factor for the development of peptic ulcer disease, gastric adenocarcinoma, and gastric lymphomas (25, 72, 87, 104). Gastric adenocarcinoma is the second leading cause of cancer-related deaths in the world, and has been associated with the presence of *H. pylori* in the stomach (72). Only a small percentage (approximately 3%) of individuals infected with *H. pylori* ever develop cancer or peptic ulcers, indicating that other factors are involved (i.e. environmental, diet, and host factors) (72). *H. pylori* infection can be eradicated by treatment with antibiotics, but most antibiotic treatment regimens require the use of multiple drugs for at least a week. Experiments involving animal models indicate that it may be possible to develop an *H. pylori* vaccine that confers protective immunity (10, 78), but thus far there is no *H. pylori* vaccine commercially available for use in humans.

Helicobacter pylori virulence factors

H. pylori has evolved sophisticated mechanisms that allow its survival and growth in the hostile environment of the human stomach. Two independent H. pylori strains have been sequenced and the overall genomic organization and predicted proteomes of the two strains are fairly similar (4). However, about 6-7% of the genes identified are present in one of the strains but not the other (4, 91). H. pylori populations have been shown to be highly diverse (39). For example, a single host can carry many closely related H. pylori strains that are derived from one parental strain by a series of mutations, chromosomal rearrangements, and recombinational events (39). Even though H. pylori diversity has made it somewhat difficult to search for bacterial factors that are associated with disease, within the past twenty years a variety of virulence factors have been described. H. pylori expresses flagella and urease, which are thought to be two of the most important factors for H. pylori colonization and survival in the human stomach (11). Flagella presumably allow the bacteria to enter and move within the gastric mucus layer (65), and urease hydrolyzes down urea into ammonia, which contributes to the acid resistance of H. pylori (55). In addition, most H. pylori strains express a variety of adhesins, as well as porins, catalase, and other virulence determinants.

Two of the most extensively studied H. pylori virulence factors are the *cag* pathogenicity island (PAI) (13) and the vacuolating cytotoxin (VacA) (43). The PAI encodes a type IV secretion apparatus that is used to translocate the H. pylori effector protein CagA (63, 86), as well as bacterial peptidoglycan into the eukaryotic cell (99). Inside mammalian cells, CagA localizes to the cell membrane and is phosphorylated by members of the Src family of tyrosine kinases (85). Phosphorylation of CagA has been

associated with the activation of the host-cell tyrosine phosphatase SRC homology 2-phosphatase (SHP-2), resulting in aberrant host-cell signaling and morphological changes characterized by extensive rearrangement of the actin cytoskeleton (6, 36). *H. pylori* peptidoglycan translocated into host cells has been shown to be recognized via Nod1 (99), an intracellular pathogen-recognition molecule specific for Gram-negative peptidoglycan (35). Activation of Nod1 by *H. pylori* results in the activation of a proinflammatory response in gastric epithelial cells (99). Another important *H. pylori* virulence factor, the vacuolating cytotoxin (VacA) (43), is the focus of this thesis work and will be described in more detail in the sections below.

Helicobacter pylori vacuolating cytotoxin (VacA)

In 1988 Leunk *et al* reported that broth-culture supernatants from *H. pylori* isolates contained a proteinaceous cytotoxin that was able to induce cytoplasmic vacuolation in eukaryotic cells (Fig. 1) (43). Subsequently, a protein with vacuolating activity named VacA was purified from *H. pylori* broth-culture supernatants (15). All clinical *H. pylori* isolates contain the *vacA* gene, but not all isolates express a cytotoxic VacA protein (approximately 50% of strains from the U.S. patients produce detectable vacuolating cytotoxic activity in a HeLa cell assay) (7, 8, 15). Variation in levels of cytotoxicity produced by different strains has been related to *vacA* allelic variation among *H. pylori* isolates (Fig. 2) (7). Two different types of VacA signal sequences (s1 and s2) and two types of VacA mid-regions (m1 and m2) have been described (7). Mosaic forms of *vacA* are thought to arise via homologous recombination among different *vacA* alleles.
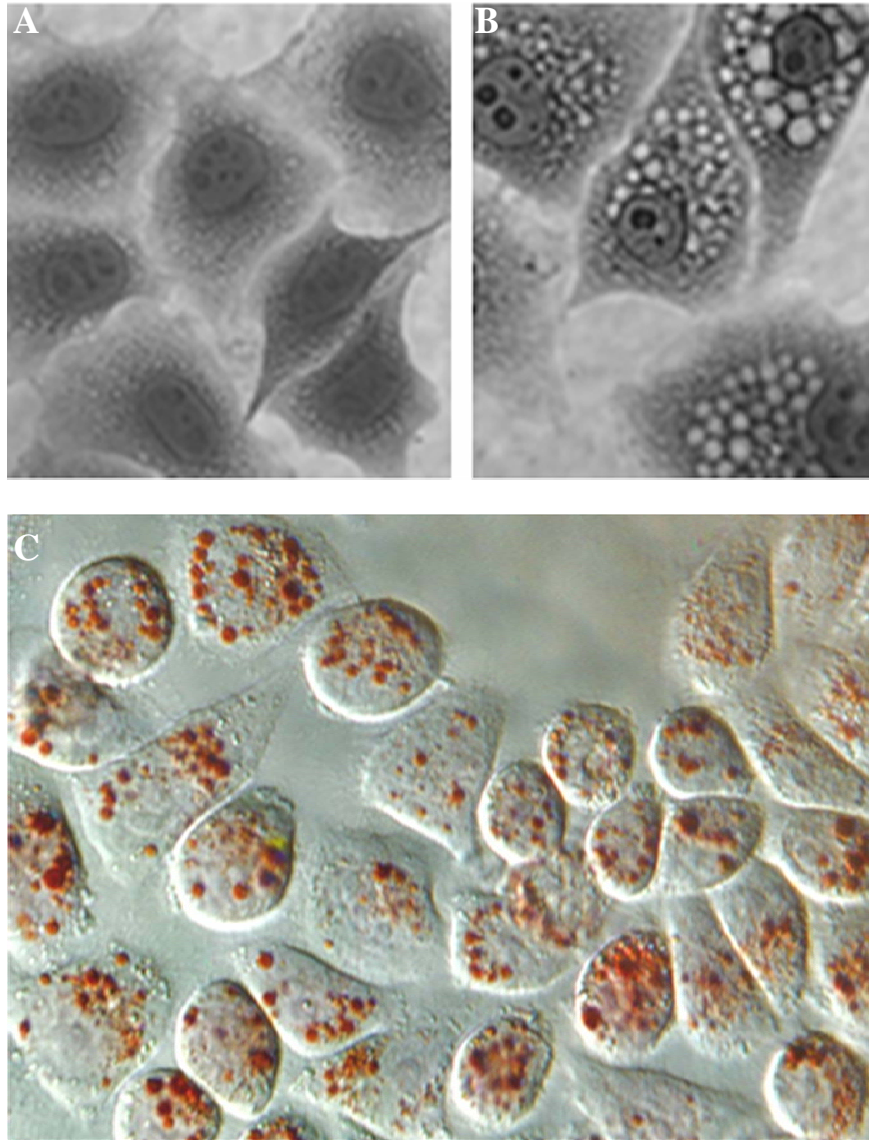
Figure 1. Cytotoxic effects of VacA on HeLa cells. HeLa cells were treated as described below, for 2 h at 37 °C, stained with crystal violet (A and B) or with Neutral Red, and then visualized by microscopy. *A*) Cells treated with buffer without VacA. *B and C*), Cells intoxicated with acid-activated VacA (5 µg/ml).

H. pylori isolates containing an s1-m1 *vacA* allele are the most toxic strains followed by *H. pylori* strains containing s1-m2 *vacA* alleles (7). The *H. pylori* isolates containing s2-m1 or s2-m2 *vacA* alleles do not exhibit detectable cytotoxic activity *in vitro*, and are infrequently associated with symptomatic disease(7).

Expression, regulation, and secretion of the mature VacA protein

Very little is known about how *H. pylori* regulates expression of the VacA protein. It has been shown that VacA expression is upregulated after initial contact of *H. pylori* with human gastric epithelial cells *in vitro* (98). Moreover, VacA expression has been shown to be induced under acidic conditions and in response to iron starvation (9, 54).

The *vacA* gene encodes a precursor 139-140 kDa protoxin (15, 18, 80, 90). The 139-140 kDa protoxin undergoes cleavage of an N-terminal signal sequence and cleavage of a C-terminal auto-transporter domain to produce a final secreted product of 88 kDa (Fig. 2) (61). The signal sequence facilitates the transport of the protoxin from the cytoplasm to the periplasm. Once in the periplasm, it is believed that the toxin is translocated to the outer membrane using its auto-transporter domain. VacA can remain associated with the surface of the bacteria, from which can be transferred directly to host cells at the bacteria-cell interface, or it can be secreted into the extracellular environment as a soluble protein (38). Analyses of the amino acid sequence of the mature secreted VacA have shown no striking similarity to any known bacterial toxin or protein.
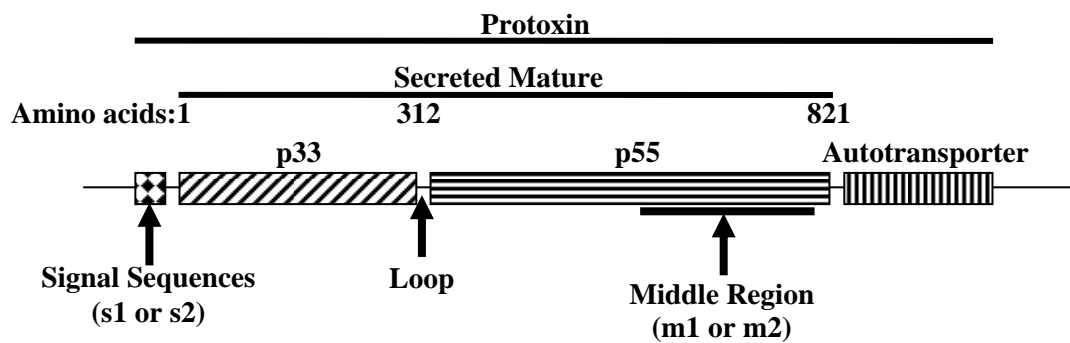
Figure 2. Schematic representation of VacA protein. The *H. pylori vacA* gene is translated as a 139-140 kDa protoxin and shows no striking similarity to any known bacterial toxin. Upon expression, this protoxin undergoes amino- and carboxyl-terminal processing to yield a mature 88-kDa secreted VacA toxin. The mature VacA toxin can be cleaved into amino- and carboxyl-terminal fragments, of 33-kDa (p33) and 55 kDa (p55), respectively (15, 61, 90).

Role of VacA in gastroduodenal disease

Experimental animal studies and epidemiological studies have suggested that VacA is an important virulence factor in *H. pylori* pathogenesis (7, 30, 90). Oral administration of sonicated extract from *H. pylori* strains containing s1-m1 VacA to mice induces pathological lesions similar to those found in human biopsies (48). In addition, oral administration of purified VacA protein to mice induces gastric ulceration (90). VacA also induces cell vacuolation *in vivo* in the stomach of animal models as well as in primary gastric cell cultures (30, 43, 48, 90). Cell vacuolation has also been observed in biopsies of infected humans (90). The importance of VacA-induced vacuolation in *H. pylori*-mediated diseases remains poorly understood. It seems likely that additional activities of VacA may be important *in vivo*.

VacA effects on mammalian cells

The *H. pylori* VacA toxin produces a wide array of structural and functional alterations in intoxicated mammalian cells (Fig. 3). The most prominent VacA effect is the formation of large vacuoles within the cytoplasm of mammalian cells, an effect observed about 90 minutes after VacA intoxication (Fig. 1 and 3) (15, 43). VacA-induced vacuoles accumulate several cell-permeable dyes, including the acidotrophic dye neutral red, which becomes trapped inside the VacA-induced vacuoles (15). The ability of VacA-induced vacuoles to accumulate neutral red provides the basis for a quantitative assay (neutral red uptake assay) which is used to assess vacuolating activity of VacA proteins (15). Vacuoles induced by VacA originate from membrane-bound

compartments of the late endocytic pathway and are characterized by the presence of V-ATPase and the small GTP-binding protein rab7 (44, 67, 68).

Another important activity of VacA is its ability to form membrane channels (19). VacA forms hexameric, anion-selective, channels upon insertion into planar lipid bilayers, and in cell membranes(Fig. 3) (19, 89). The formation of VacA channels in the plasma membrane of cells results in increased permeability to small anions, including chloride, bicarbonate, and pyruvate, with consequent membrane depolarization (69). VacA membrane channels have been shown to be responsible for VacA vacuolating cytotoxic activity (Fig. 3)(93, 100). It has been proposed that VacA causes cell vacuolation by forming anion-selective channels in the membrane of endocytic compartments (Fig. 3)(44). VacA also increases transepithelial permeability of polarized epithelial monolayers to ions, such as iron, and nickel, as well as small sugars and urea (Fig. 3) (69, 74, 92). The ability of VacA to increase the transepithelial permeability of these molecules might be beneficial for *H. pylori*, since iron, nickel, and sugars can be used as nutrients, and hydrolysis of urea contributes to the acid resistance of *H. pylori*.

VacA seems to have the ability to induce a variety of detrimental effects on host cells *in vitro* (see Fig. 3) (58, 70). Other VacA effects on mammalian cells include i) apoptosis (17), presumably by disrupting the mitochondria membrane potential, resulting in cytocrome c release (31, 105, 106); ii) clustering and redistribution of late endocytic compartments (44, 67, 68), which might result in interference with class II antigen presentation (56), impairment of endocytic trafficking (79), and disruption of phagosome maturation in macrophages (3, 115); iii) detachment of epithelial cells from the basement membrane, an effect dependent on VacA interaction with RPTP-β (see below) (30); iv)

activation of mitogen-activated protein kinase (MAPK) p38 and extracellular signal-regulated kinase 1/2 (Erk1/2), effects dependent on VacA binding to the cell surface but not on VacA channel activity (12, 59, 88); and, v) inhibition of activation-induced proliferation of T lymphocytes (this topic will be described in more detail in CHAPTER IV) (12, 34). Many of these effects have been shown to be dependent on the capacity of VacA to form anion-selective membrane channels (17, 88, 100, 105, 106). Potentially several of these effects of VacA are relevant to *H. pylori* pathogenesis *in vivo*.
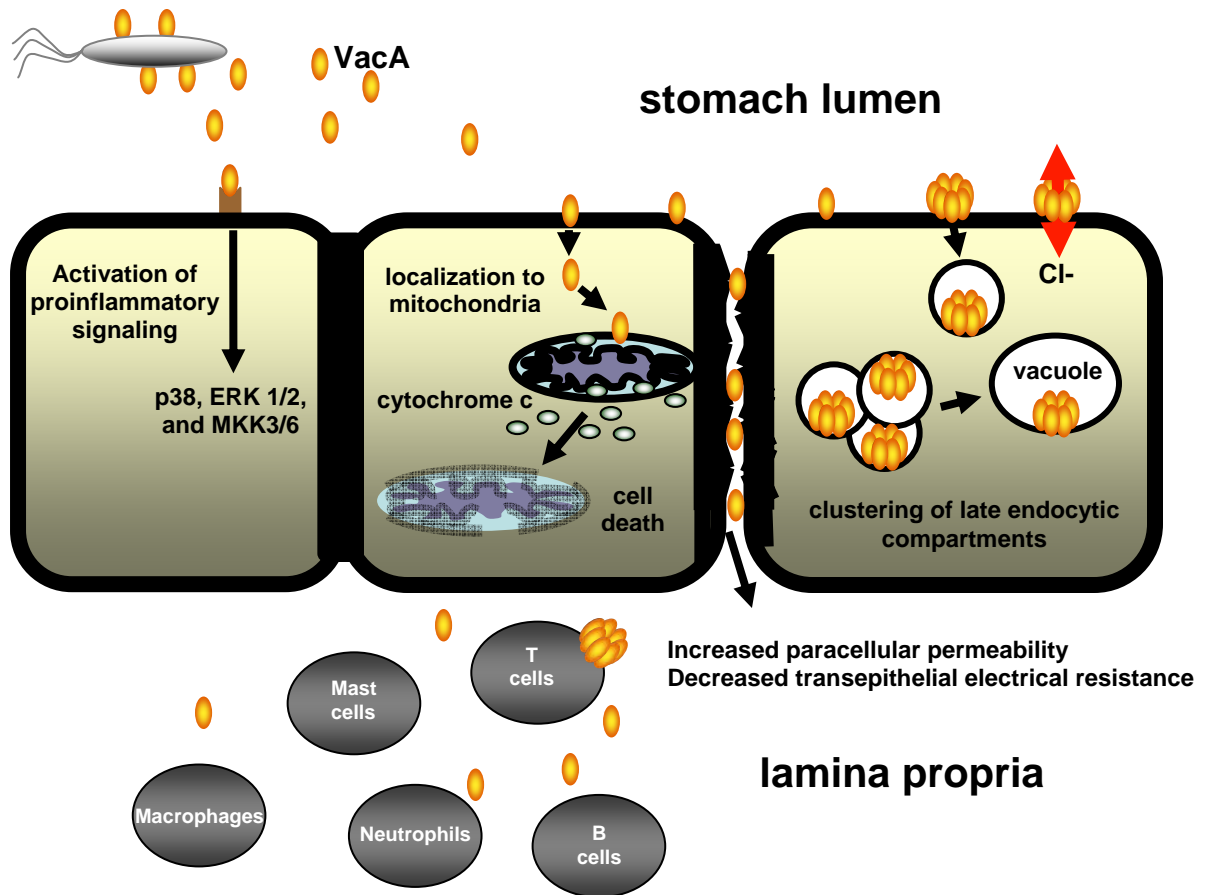
Figure 3. Model of VacA effects on mammalian cells. VacA can be found bound to *H. pylori* surfaces or secreted. Secreted VacA binds to putative cellular receptor(s) inducing the activation of proinflammatory signals. VacA has been shown to activate the mitogen-activated protein kinases (MKK) 3/6, p38, and the extracellular signal-regulated kinase (ERK) 1/2 in gastric epithelial cells as well as in immune cells. VacA also is internalized into host cells. Internalized VacA can localize to late endosomes or mitochondria. VacA interactions with mitochondria lead to reduced mitochondrial membrane potential and cytochrome c release, resulting in cell death. VacA also induces increased paracellular permeability and reduced transepithelial electrical resistance in polarized cells. These effects might allow VacA to migrate from the stomach lumen to the lamina propia, were VacA can interact with immune cells. VacA can also interact with epithelial cell surfaces to form anion-selective membrane channels allowing chloride transport across the cell membrane. Endocytosed VacA causes clustering of late endocytic compartments, and swelling of these clustered endosomes results in the formation of VacA-induced vacuoles.

VacA receptors and internalization mechanism

In order for VacA to exhibit cytotoxic effects, it must bind to the plasma membrane of cells and then undergo internalization (53). The process of VacA binding to mammalian cells has been studied for several years, but whether there is a specific VacA receptor is still controversial. It is thought that the carboxy-terminal region of VacA is responsible for binding to a putative receptor on the epithelial cell surface (32, 76, 101, 103). Indeed, several cell-surface receptors for VacA have been reported, including two receptor protein tyrosine phosphatases (RPTP α and β) (30, 109), the epidermal growth factor receptor (83), and heparan sulfate (97), a glycosaminoglycan present on the surface of most eukaryotic cells. VacA binding to RPTP molecules has been studied in the most detail, and has been shown to be dependent on post-translation modifications of these receptors, suggesting that VacA might recognize sialic acid and sugar moieties on these proteins (109, 110). Cells resistant to VacA cytotoxic effects become sensitive when transfected with RPTP-β expressing-plasmids, suggesting that RPTP-β is involved in VacA intoxication (66). Similarly, VacA-sensitive cells treated with RPTP-α or β morpholino antisense oligonucleotide before intoxication are resistant to VacA cytotoxicity (66, 109). Interestingly, in mice that do not express RPTP-β in the stomach, VacA still binds to gastric cells and induces vacuolating cytotoxic activity, suggesting that VacA can use more that one receptor to exhibit its cytotoxic effects on mammalian cells (30). In addition, VacA localizes to sphingolipid-cholesterol-rich-membrane microdomains (lipid rafts) on the surface of mammalian cells (81). It might be possible that VacA binds to protein receptors as well as to lipids on the surface of host cells.

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Following binding of VacA to the plasma membrane, the toxin is internalized by cells (53), a process inhibited by the disruption of lipid rafts (33, 42, 71, 81), suggesting that VacA may use lipid rafts for binding and internalization. The exact mechanism employed by VacA to gain access to the cytoplasm of mammalian cell is largely unknown. It has been proposed that VacA binds to a receptor which undergoes endocytosis, allowing the toxin to be internalized via a clathrin-independent pathway (71, 77). It might be possible that VacA binds to a cell surface molecule(s), promoting the aggregation of VacA in lipid rafts on the cell surface, which in turn, induces VacA insertion into the cell membrane and pore-formation, followed by internalization of these VacA complexes via an actin-dependent endocytic process.

p33 and p55 VacA domains

The mature secreted 88 kDa VacA toxin can undergo spontaneous proteolytic degradation into fragments that are about 33 and 55 kDa in mass (16, 32, 61, 90). This degradation has been observed especially when preparations of the purified toxin are stored for prolonged periods (32, 90). Whether the generation of the 33- and 55 kDa VacA fragments is the result of auto-proteolysis is not currently known. It has been presumed that the two fragments (termed p33 and p55) represent two domains or subunits of VacA (46, 76, 90). Several lines of evidence indicate that amino acid sequences located within a hydrophobic region near the N-terminus of the p33 domain are required for the formation of anion-selective membrane channels (49, 52, 100, 112), and that amino acids within the p55 domain are responsible for VacA binding to mammalian cells (32, 76, 101, 103). Interestingly, when transiently expressed within mammalian cells,

neither the p33 nor the p55 VacA domain is sufficient for vacuolating cytotoxic activity

(113). In contrast, when the p33 and p55 are transiently co-expressed within mammalian

cells, they complement each other, resulting in vacuolating activity (113).

VacA oligomeric structure

Over the past decade, several lines of evidence have suggested the occurrence of

homotypic interactions among 88 kDa VacA proteins (15, 50, 76, 90, 100, 107, 111).

The first evidence suggesting oligomerization of VacA proteins was the observation that

purified VacA from *H. pylori* broth culture supernatant assembles into structures of

~1,000 kDa in mass under non-denaturing conditions (15). Ultrastructural studies have

shown that VacA complexes have a flower-like shape structure and are composed of

anywhere from 6 to 14 VacA monomers (Fig. 4) (1, 16, 46). In addition, it has been

shown that these flower-shaped structures disassemble into VacA monomers after

exposure to acid or alkaline pH and then reassemble into the flower-shaped structures

after neutralization (16). Additional evidence of VacA assembly into oligomeric

structures has come from experiments in which two different VacA toxins have been

shown to form mixed oligomeric structures (50, 100). It is believed that VacA assembly

into oligomeric structures is required for the toxin's cytotoxic affects.
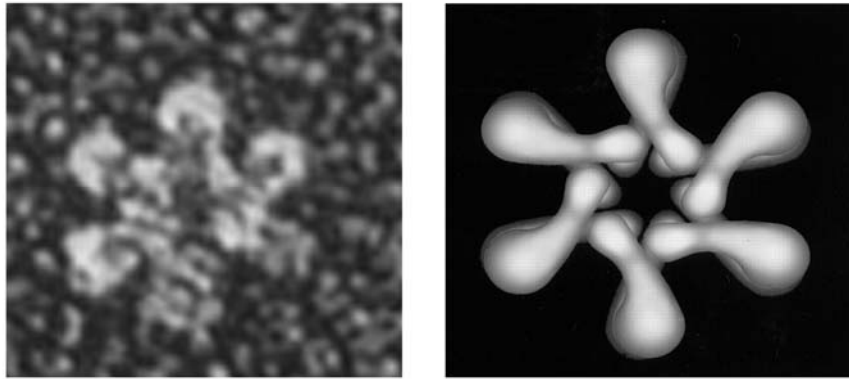
Figure 4. Flower-like VacA structure. *A*) Purified VacA molecules imaged by deep-etch electron microscopy. Rotary replica of a purified and freeze-dried VacA flower purified from broth culture supernatants of tox$^+$ *H. pylori* strain 60190 (s1/m1 VacA). *B*) A structural model of VacA based on the flower view, interpreted to be a duodecamer composed of two hexameric flat forms interlocked face-to-face. Pictures were modified from Cover *et al* (16).

Research significance and specific aims

<u>Study of VacA oligomerization</u>

Experimental and epidemiological studies have suggested that the vacuolating cytotoxin (VacA) is an important virulence factor in *H. pylori* pathogenesis, and that VacA contributes to the development of stomach and duodenal ulcers (7, 30, 90). VacA assembly into oligomeric structures able to form membrane channels is thought to be required for most VacA-induced effects on mammalian cells. This hypothesis is supported by electron microscopy studies in which VacA associated with membranes appears as flower-shaped structures (1), as well as electrophysiologic studies in which the kinetics of membrane channel formation suggest that the VacA channel is a hexamer (40).

Previous studies have demonstrated that the mature secreted VacA toxin can undergo spontaneous proteolytic degradation to yield two fragments (p33 and p55) (16, 90). It has been suggested that these two fragments represent two distinct VacA functional domains, but in the absence of a VacA crystal structure, the relevant features of these two putative domains remain poorly characterized. The study of the proccess of VacA assembly into oligomeric strucutures and the role of p33 and p55 domains in VacA assembly has been hindered by the lack of over-expression systems that allow the production of functionaly active recombinant VacA proteins. Furthermore, VacA domains involved in toxin binding and internalization into mammalian cells have not yet been decribed in any detail. Thus, the main goal of this thesis was to identify functional

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domains of VacA, and in particular, to characterize the role of p33 and p55 domains in the process of VacA oligomerization and intoxication of mammalian cells.

Proteins normally exist in nature in one of two generally different environments, either as membrane bound or water-soluble forms. Interestingly, pore-forming proteins have evolved and acquired a remarkable ability to be in either a soluble or membrane bound state. Most of these pore-forming proteins require protein oligomerization for the transition from a soluble to a membrane-bound state. Examples of pore-forming proteins include perforin and Bcl-2 family members, as well as several bacterial toxins. Pore-forming toxins are interesting not only because they might play an important role in the ability of bacteria to cause disease, but also because, like VacA, they could be used as models to study how pore-forming proteins undergo the transition from water-soluble to membrane bound forms. Furthermore, analysis of the processes by which pore-forming toxins assemble into oligomeric structures may ultimately lead to the development of therapeutic inhibitors that block the oligomerization and the activity of these toxins *in vivo*.

VacA effects on primary human T lymphocytes

Another important area of research is to try to identify the *in vivo* function or role of VacA in *H. pylori* infection. *H. pylori* can persistently colonize the human gastric mucosa for decades despite the development of gastric mucosal inflammation and specific antibody production. Several lines of evidence indicate that CD4$^+$ T cells are critical for protection against *H. pylori* infection (2, 22, 41, 45). Thus, it seems possible that immune evasion strategies of *H. pylori* virulence factors may involve the inhibition or modulation of T cell immunity. VacA is an attractive candidate to modulate the

immune system since this protein is released from the bacteria into the extracellular environment (15). This hypothesis is further supported by the ability of VacA to inhibit activation of Jurkat T cells (a human T cell lymphoma/leukemia cell line) as well as human peripheral blood lymphocytes (PBL) (this topic will be described in more detail in CHAPTER IV) (12, 34). Furthermore, VacA has been shown to specifically inhibit the Ii-dependent pathway of antigen presentation mediated by major histocompatibility complex (MHC) class II (56). Together, these studies indicate that VacA is capable of interfering with various steps in the immune response to *H. pylori*. Infection with *H. pylori* causes the infiltration of neutrophils, T- and B-lymphocytes, plasma cells and macrophages to the site of infection, resulting in the extensive gastric mucosal inflammation observed in infected individuals (22, 41). Most of the studies of VacA-mediated effects on immune cells have been done using transformed cell lines (12, 34, 56). It is important to investigate VacA effects on primary human cells, which presumably are more appropriate for analyzing physiological responses to this toxin *in vivo*. Therefore, another goal of this thesis was to investigate the effects of VacA on primary human CD4$^+$ T cells. Furthermore, VacA might serve as a tool like many other bacterial toxins (e.g. diphtheria, aerolysin, tetanus, and botulinum) to further dissect cell processes that were previously uncharacterized.

CHAPTER II

INTERACTIONS BETWEEN P33 AND P55 DOMAINS OF THE
HELICOBACTER PYLORI VACUOLATING CYTOTOXIN

Introduction

The VacA toxin secreted by *Helicobacter pylori* is considered to be an important virulence factor in the pathogenesis of peptic ulcer disease and gastric cancer (7, 30, 90). VacA monomers self-assemble into water-soluble oligomeric structures comprised predominantly of 6 to 14 VacA monomers (1, 16, 46). Assembly of VacA into oligomeric structures is required for cytotoxic effects, in particular for the formation of membrane channels (1, 40). Thus far, the process by which VacA assembles into higher order structures has not been studied in any detail. Therefore, the goal of this study was to characterize interactions that may mediate assembly of VacA into oligomeric structures. The mature secreted 88 kDa VacA toxin can undergo spontaneous proteolytic degradation into fragments that are about 33 kDa and 55 kDa in mass (16, 90). It has been presumed that the two fragments (termed p33 and p55) represent two domains or subunits of VacA (16, 90). The role of p33 and p55 domains in VacA assembly has not yet been study in any detail. To facilitate the study of VacA assembly, a yeast two-hybrid system was used to investigate whether the p33 and p55 domains physically interact.

Methods

Bacterial and yeast strains, media, and growth condition: H. pylori strain 60190
(ATCC 49503) was grown on Trypticase soy agar plates containing 5% sheep blood at 37
^{o}C in ambient air containing 5% CO_2. Liquid cultures were grown in sulfite-free brucella
broth supplemented with either 5% fetal bovine serum or 0.5% activated charcoal.
Escherichia coli XL1-Blue (Stratagene) was used for plasmid propagation and was
grown on Luria-Bertani (LB) broth or LB agar at 37 ^{o}C. Yeast two-hybrid experiments
were performed with *Saccharomyces cerevisiae* strain YRG-2 (*MAT* α *ura*3-52 *his*3-200
*ade*2-101 *lys*2-801 *trp*1-901 *leu*2-3,112 *gal*4-542 *gal*80-538 LYS2::UAS$_{GAL1}$ -
TATA$_{GAL1}$-HIS3 URA3::$_{UASGAL4\ 17mers(x3)}$-TATA$_{CYC1}$-*lac*Z) (Stratagene). Yeast strains
were grown in rich medium (yeast extract, peptone, dextrose [YPAD]) or in synthetic
defined (SD) minimal medium (supplemented with required amino acids and glucose) at
30 ^{o}C as described in the GAL4 Two-Hybrid Phagemid manual (Stratagene).

Construction of yeast two-hybrid vectors containing vacA fragments: vacA
sequences encoding the wild-type p33 and p55 VacA domains were PCR-amplified from
genomic DNA of *H. pylori* strain 60190 and cloned into plasmids encoding the
transcription-activation domain (pAD) and/or the DNA-binding domain (pBD) of the
GAL4 Two-Hybrid Phagemid system (Stratagene). To facilitate the introduction of in-
frame deletion mutations into the pAD33 plasmid, *vacA* sequences encoding p33 mutant
domains were PCR-amplified from genomic DNA of previously described *H. pylori*
strains harboring the specific in-frame *vacA* deletions, and cloned into the pAD vector.
vacA sequences encoding p55 fragments were PCR-amplified from genomic DNA of *H.*

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pylori strain 60190 and were cloned into the pBD vector. The entire *vacA* fragment in each p33 and p55 encoding plasmid was analyzed by nucleotide sequence analysis in order to verify that no unintended mutations had been introduced.

Yeast methods: Yeast strains were co-transformed with 400 ng of individual plasmids using the lithium acetate method and cultured on SD medium supplemented with the required amino acids and glucose at 30 °C as described in the GAL4 Two-Hybrid Phagemid manual (Stratagene). For a positive protein-protein interaction control, we co-transformed the yeast with pBD-WT and pAD-WT plasmids, which encode fusion proteins consisting of amino acids 132-236 of wild-type lambda cI, fragment C, together with either the GAL4- BD or AD, respectively. For a negative control (i.e., two proteins that do not interact), we used the pAD-WT plasmid co-transformed with a pBD-pLamin C plasmid, which expresses the BD of GAL4 fused to amino acids 60-230 of human lamin C. We also used plasmids pAD-Mut and pBD-Mut, which encode a mutated (E233K) lambda cI, fragment C, fused to either the GAL4- AD or BD. The cI-E233K mutation interferes with the interaction between the cI monomers, resulting in a protein-protein interaction that is weaker than that of wild-type proteins. Plasmids expressing positive and negative interaction control proteins were obtained from Stratagene.

One colony of each co-transformant was grown in 2 ml of SD medium containing 2% (w/v) glucose and lacking Trp and Leu (SD-Leu,-Trp) at 30 °C with aeration for ~24 h. Cultures were then normalized to an OD_{600} of about 0.26 in a final volume of 100 μl of SD-Leu,-Trp,-His broth, and 10-fold serially diluted into SD-Leu,-Trp,-His broth in a micro-titer plate. Diluted cultures (5 μl) were seeded on duplicate SD-Leu,-Trp plates

(selection for plasmids) and SD-Leu,–Trp,–His plates (selection for protein-protein interactions) and incubated at 30 °C for 3-10 days.

To confirm the occurrence of protein–protein interactions, a β-galactosidase liquid assay was performed using the Yeast β-Galactosidase Assay Kit as described by the manufacturer (Pierce). Briefly, individual co-transformants were grown in 2 ml SD - Leu,-Trp medium containing 2% (w/v) glucose at 30 °C with aeration for ~16-24 h until cultures reached mid-log phase (OD_{600} 0.4-0.5). 150 μl aliquots of the cultures were then mixed with 150 μl of the working solution (equal volume of 2X β-Galactosidase Assay Buffer and Y-PER^{TM} Reagent) and then incubated at 37 °C until solutions turned yellow (approximately 1-4 h), at which time reactions were stopped by the addition of 175 μl of β-Galactosidase Assay Stop Solution. The time required for yellow color development was recorded for each tube. The OD_{410} of clarified reaction supernatants (200 μl) were measured in a 96-well plate and the β-galactosidase activity was calculated by using the Miller equation as described in the Yeast β-galactosidase Assay Protocol (Pierce). The values presented are the average of at least three independent co-transformants (means $\pm$ S.D.). Statistical significance was analyzed using Student's *t* test.

Introduction of the FLAG-tag epitope and enterokinase-site into vacA: To modify the *vacA* gene so that it encoded a VacA protein containing a FLAG-tag (DYKDADDD<u>K</u>) and an enterokinase cleavage site (after the underlined K), complementary primers encoding the FLAG epitope were annealed and ligated into the unique *Stu*I site of plasmid pA178(50). The resulting plasmid, pA178-FLAG, contained a sequence encoding the FLAG epitope in the proper orientation inserted between amino

acids 317 and 318 of VacA. Plasmid pA178-FLAG was then used to transform *H. pylori* strain VM022 (100), and transformants were selected by growth on 5.5% sucrose plates (100). Analysis of a single transformant (*H. pylori* VM088) by PCR and by DNA sequence analysis confirmed that the sequence encoding the FLAG epitope and the enterokinase cleavage site had been introduced into the desired location.

Purification of oligomeric forms of VacA: VacA was purified from broth culture supernatants of *H. pylori* (16). Broth culture supernatant proteins were concentrated by precipitation with a 50% saturated solution of ammonium sulfate and resuspended in phosphate-buffered saline. Oligomeric VacA (~1,000 kDa) was purified by gel-filtration chromatography using a Superose 6HR 16/50 column. To analyze the oligomeric state of the enterokinase-treated FLAG-VacA, 40 μg of cleaved toxin was applied to a Superose 6HR 16/50 column and fractions (2 ml each) were collected. Fractions were analyzed by immunoblot analysis with an anti-VacA serum.

Proteolysis of FLAG-VacA with enterokinase: Purified FLAG-VacA was proteolytically cleaved with enterokinase as described by the Recombinant Enterokinase Kit manual (Novagen). Briefly, 10 μg of purified FLAG-VacA was incubated with 1 unit of enterokinase and 5 μl of 10X enterokinase cleavage buffer in a final volume of 50 μl for ≥16 h at room temperature. For mock treatment, FLAG-VacA was treated in the same manner, but without enterokinase. Proteolysis of the full-length FLAG-VacA was assessed by immunoblot analysis using an anti-VacA serum or the M2 monoclonal anti-FLAG antibody (Sigma).

Cell culture and vacuolating assays: HeLa cells were grown in minimal essential medium (modified Eagle's medium containing Earle's salts; MEM) supplemented with 10% fetal bovine serum. For vacuolating assays, HeLa cells were seeded (1.5×10^4) into 96-well plates 24 h prior to each experiment. Protein concentrations of the purified VacA toxins were determined by using a Micro-BCA assay (Pierce). Purified VacA toxins (standardized by protein concentration) were incubated with cells in serum-free medium containing 10 mM ammonium chloride (15). Purified VacA preparations (VacA, FLAG-VacA and cleaved FLAG-VacA) were acid-activated by adjusting them to pH 3 by the addition of 250 mM hydrochloric acid before they were added to cell culture wells (21, 53). After 24 h incubation of VacA toxins with the cell monolayers, cells were examined by inverted light microscopy. Toxins that induced vacuoles in more than 50% of the cells were scored positive for vacuolating cytotoxic activity. Vacuolating activity was quantified by a neutral red uptake assay and the data presented as OD_{540} values (means $\pm$ S.D.).

p33 and p55 VacA domains interact in a yeast two-hybrid system

The 88 kDa VacA monomers commonly undergo spontaneous degradation into p33 and p55 VacA domains (Fig. 5) (16, 90). To investigate potential interactions between these two VacA domains, we investigated whether they were able to interact in a yeast two-hybrid system (GAL4 Two-Hybrid Phagemid; Stratagene) (28). In the yeast two-hybrid system, the yeast GAL4 transcription activator protein has been divided into two separate functional domains(47): (i) the transcription-activation domain (AD) present on plasmid pAD-GAL4-2.1 (pAD), which encodes for the *LEU2* gene as a selectable marker, and (ii) the DNA-binding domain (BD) present on the plasmid pBD-GAL4-Cam (pBD), which encodes for the *TRP1* gene as a selectable marker. Proteins of interest are then fused to either the GAL4-AD or the BD domains and if two fusion proteins interact, they will bring in close proximity the GAL4 transcription-activation domain and the GAL4 DNA-binding domain to the GAL4/GAL1 promoter, which in turn, will initiate the transcription of the *HIS3* and *lacZ* reporter genes. Protein-protein interactions are then detected by the ability of co-transformed yeast cells to grow in selective medium lacking Leu, Trp, and His (SD-Leu,-Trp,-His) and by production of β-galactosidase activity.
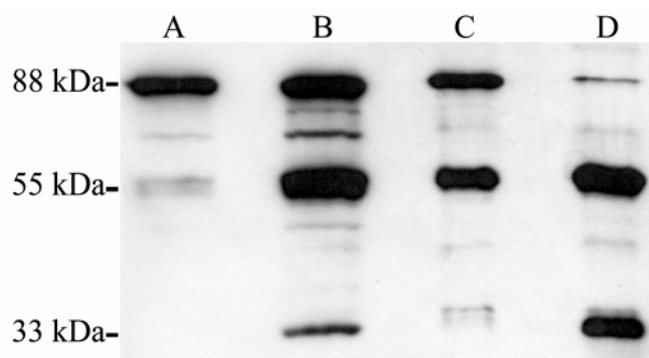
Figure 5. Proteolytic degradation of VacA into 33 and 55 kDa fragments. Proteins precipitated from broth culture supernatant of *H. pylori* 60190 by a 50 % saturated solution of ammonium sulfate (lanes A and B) or purified VacA (lanes C and D) from *H. pylori* 60190 were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and immunobloted with polyclonal anti-VacA serum. These preparations exhibit varying degrees of spontaneous VacA proteolysis into p33 and p55 fragments.

vacA sequences encoding the p33 and p55 VacA domains were cloned into plasmids encoding the GAL4-AD (pAD) and the BD domains (pBD), generating plasmids pAD33, pBD33, pAD55 and pBD55. To test whether interactions between p33 and p55 VacA domains could be detected in this system, we co-transformed all four possible combinations of the p33 and p55 yeast two-hybrid plasmids into the yeast reporter strain (Fig. 6A). Serial dilutions of co-transformed yeast were then tested for their ability to grow on synthetic defined (SD) minimal medium lacking Leu and Trp (SD-Leu and -Trp), as well as SD medium lacking Leu, Trp, and His (SD-Leu, -Trp, and -His). All of the co-transformed yeast grew on the former media, which confirmed that the transformation had been successful and provided an indication of the relative number of co-transformed yeast cells present in each dilution. Yeast co-transformed with plasmids encoding p33 and p55 fusion proteins grew in SD medium lacking Leu, Trp, and His, indicating that the p33 and p55 domains interact in this system (Fig. 6B). In contrast, yeast co-transformed with plasmids encoding p33/p33 or p55/p55 fusion proteins did not grow on SD medium lacking Leu, Trp and His, suggesting that they did not interact (Fig. 6B). The ability of p33 and p55 to interact was further confirmed by the analysis of β-galactosidase activity, which reflects the activation of the *lacZ* reporter gene (Fig. 6C). The p33/p55 interaction was independent of the protein fused (AD or BD) to the p33 or p55 fragment, since both combinations of tested plasmids (pAD33/pBD55 and pAD55/pBD33) were able to activate the *HIS3* and *lacZ* reporters (Fig. 6; panels B and C).
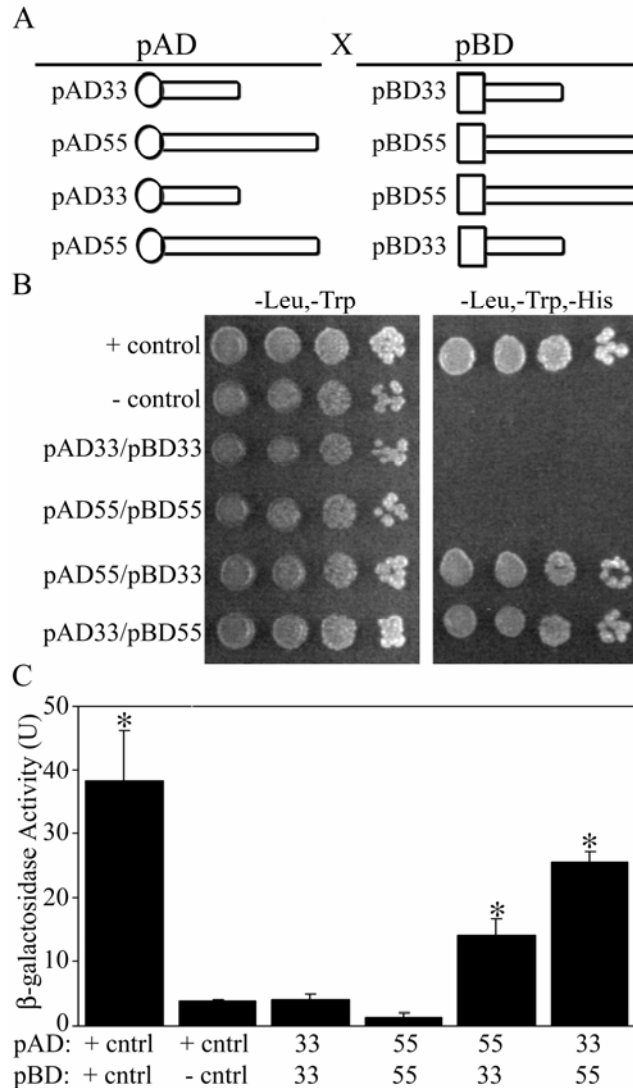
Figure 6. Interaction of VacA fragments in the yeast two-hybrid system. *A*) Representation of the different p33 and p55 fusion proteins and plasmid combinations used in the co-transformation experiments. *B*) Yeast cells were co-transformed with the plasmids depicted in panel A, or with controls plasmids described in the Methods section. Co-transformed yeast-cells (normalized based on OD) were 10-fold serially diluted and identical inocula were plated on two types of SD agar plates. All of the co-transformed yeast grew on SD-Leu,-Trp plates. Protein-protein interactions were assessed by growth of the co-transformed yeast cells on SD-Leu,-Trp,-His plates. *C*) β-galactosidase liquid culture assay of the co-transformed yeast-cells. Activation of the protein-protein interaction reporters were only detected when yeast were co-transformed with plasmids encoding wild-type p33 together with a plasmid encoding wild-type p55. Results represent the mean ± S.D. from triplicate determinations, each representing a separate colony. * = P (<0.05) when compared to the negative control. + cntrl = positive control, - cntrl = negative control.

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Disruption of the p33/p55 interaction correlates with the lack of VacA oligomerization and vacuolating activity

In a previous study, several *H. pylori* mutant strains containing in-frame deletion mutations in the *vacA* gene were constructed and analyzed (Fig. 7A) (100). Results from this previous study indicated that a mutant VacA protein with a deletion of amino acids 6-27, VacA-(Δ6-27), could assemble into water-soluble oligomeric structures, and suggested that VacA proteins containing Δ28-108, Δ56-83, Δ85-127, or Δ112-196 deletion mutations were defective in the capacity for oligomer assembly (Fig. 7A) (100). We hypothesized that the latter mutations might disrupt the p33/p55 interaction, and that such a defect might account for the failure of these VacA mutant proteins to form oligomeric structures. In order to directly test this hypothesis, we introduced these same deletion mutations (i.e., Δ6-27, Δ28-108, Δ56-83, Δ85-127 and Δ112-196) into the pAD33 plasmid. Wild-type pAD33 and pADΔ6-27p33 were each able to activate both the *HIS3* and *lacZ* reporter genes when co-transformed with wild-type pBD55, whereas the other pAD33 mutants failed to activate the reporter genes when co-transformed with wild-type pBD55 (Fig. 7; panels B and C). Thus, mutations that impair p33/p55 interactions also block oligomerization of VacA secreted by *H. pylori* (100).
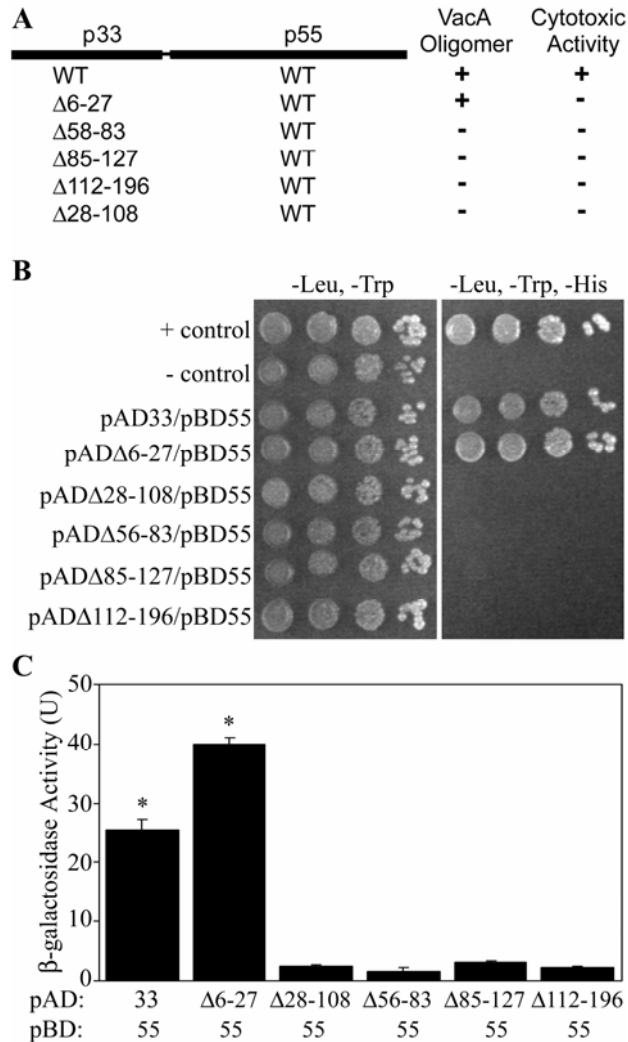
Figure 7. Interaction between wild-type p55 and p33 mutant VacA fragments. *A*) Diagram of in-frame deletion VacA mutant proteins produced in *H. pylori*. Oligomer formation of full-length VacA toxins secreted by *H. pylori* was analyzed by determining whether these proteins eluted as large oligomeric structures (~1,000 kDa) from a Superose 6 HR 16/50 FPLC column. Cytotoxic vacuolating activity of VacA toxins was assessed by intoxicating HeLa cells. *B*) Co-transformed yeast-cells (normalized based on OD) were 10-fold serially diluted and identical inocula were plated on two types of SD plates. Protein-protein interactions were assessed by growth of the co-transformed yeast cells on SD-Leu, -Trp, -His plates. *C*) β-galactosidase liquid culture assay of the co-transformed yeast-cells. Activation of the protein-protein interaction reporters were only detected when yeast were co-transformed with plasmids encoding wild-type p-33 or Δ6-27p33 together with a plasmid encoding wild-type p55. Results represent the mean $\pm$ S.D. from triplicate colonies. $*$ = P (<0.05) when compared to the negative control.

The N-terminus of the p55 domain is the minimum p55 fragment
able to interact with p33

To further map putative amino acid sequences involved in the p33/p55 interaction, we generated different p55 fragments (i.e., encoding amino acids: 313-700, 313-550, 313-478, 550-821, and 479-821) and cloned them into the pBD vector (Fig. 8A). These new fusion proteins were tested for their ability to interact with wild-type p33 as described above. Yeast co-transformed with the pAD33 plasmid and plasmids expressing p55 fragments containing amino acids 313-700, 313-550, or 313-478 were able to grow on SD-Leu,-Trp,-His, whereas yeast co-transformed with the pAD33 plasmid and plasmids expressing p55 fragments containing amino acids 479-821 or 550-821 were not able to grow (Fig. 8B). The former group of co-transformed yeast grew slowly on SD-Leu,-Trp,-His plates and expressed a relatively low level of β-galactosidase activity (Fig. 8; panel B and C). This suggests that the interactions between the 313-700, 313-550 and 313-478 p55 fragments and p33 are weaker than the interaction between full-length wild-type p55 and p33. When we co-transformed yeast with a mutated version of the positive control plasmids (Mutant + control described in the Methods section) known to encode proteins that interact with relatively low affinity, a similar low level of β-galactosidase activity was detected (Fig. 8C). The data obtained with these mutant control plasmids supports our conclusion that the p33 interacts weakly with 313-700, 313-550, and 313-478 p-55 fragments.
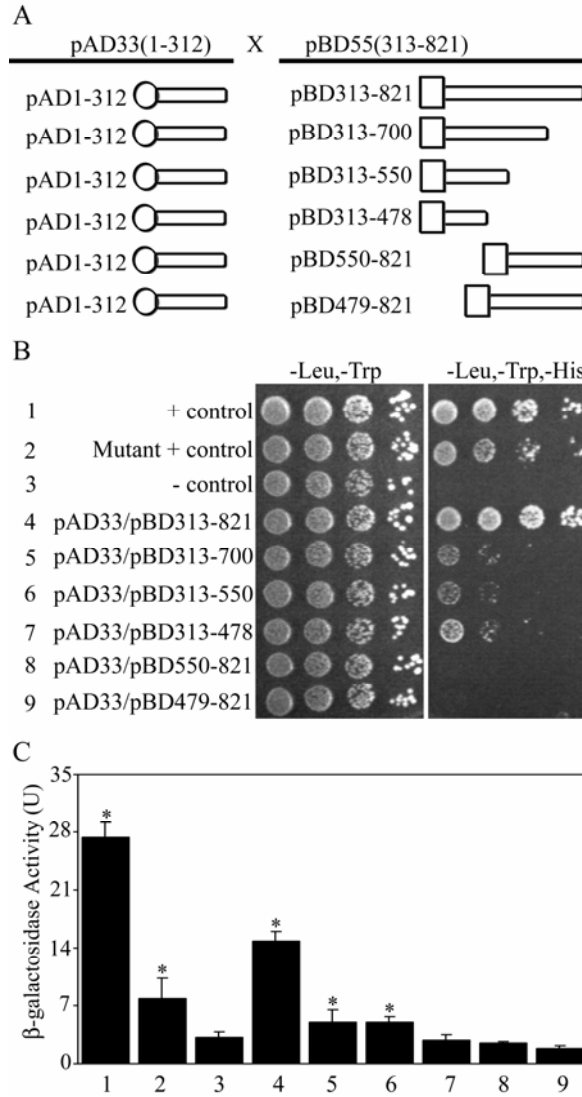
Figure 8. Interaction between wild-type p33 and fragments of p55. (A) Representation of the different p55 fragments used in the co-transformation experiment. (B) Yeast-cells co-transformed with the pBD55 fragments depicted in panel A and wild-type pAD33 (normalized based on OD) were 10-fold serially diluted and identical inocula were plated on two types of SD agar plates. (C) β-galactosidase liquid culture assay of the co-transformed yeast-cells. Protein-protein interactions were assessed by growth of the co-transformed yeast cells on SD-Leu,-Trp,-His plates and by a β-galactosidase liquid culture assay. Sample numbers in panel C are identical to those in panel B. Wild-type p33 was able to interact with p55 fragments containing amino-acids 313-700, 313-550 and 313-478 but not with amino acids 550-821 or 479-821. β-galactosidase liquid culture assay results represent the mean $\pm$ S.D. from triplicate colonies. * = P (<0.05) when compared to the negative control.

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Proteolytic cleavage of a modified VacA protein results in the generation of the p33 and p55 domains

We sought to confirm the finding that p33 interacts with p55 using VacA purified from *H. pylori.* Several previous studies have demonstrated that VacA proteins produced by *H. pylori* can degrade into 33 kDa and 55 kDa fragments (p33 and p55 respectively) (16, 90). However, it remains unclear whether proteolytic cleavage of the 88 kDa VacA protein into p33 and p55 fragments alters VacA cytotoxic activity or alters its oligomeric structure. One reason why this issue has not yet been addressed in any detail is that it has been difficult to experimentally induce the desired cleavage. VacA preparations commonly undergo partial proteolytic degradation during storage (Fig. 5), but complete proteolysis of the 88 kDa VacA protein into smaller fragments occurs uncommonly. Furthermore, spontaneous proteolytic degradation of VacA often involves cleavage at multiple sites.

In order to test experimentally whether cleavage of VacA into p33 and p55 domains alters its activity, we sought to develop a system in which a protease could be used to induce proteolytic cleavage at a specific site located between the p33 and p55 domains. To accomplish this, we constructed a modified *H. pylori* strain in which an oligonucleotide encoding the FLAG-tag epitope and an enterokinase cleavage site was inserted into the *vacA* allele of *H. pylori* 60190 resulting in *H. pylori* VM088 strain (Fig. 9).
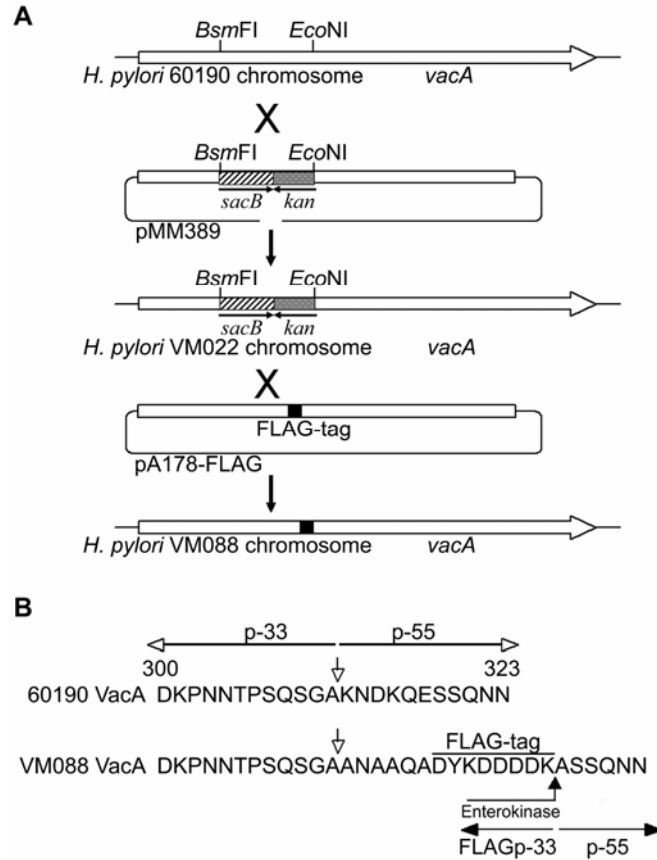
Figure 9. Construction of *H. pylori* VM088 using a *sacB*-based counter-selection approach. (A) The pMM389 plasmid contains a *vacA* fragment from *H. pylori* strain 60190 and a *sacB / kan* cassette. Transformation of *H. pylori* 60190 with pMM389 yielded a kanamycin-resistant transformant designated *H. pylori* VM022. *H. pylori* VM022 was transformed with pA178-FLAG plasmid, transformants selected on sucrose-containing medium, and a strain (*H. pylori* VM088) with a sequence encoding a FLAG-tag with an enterokinase site inserted into the *vacA* gene was thereby obtained. (B) VacA spontaneously degrades into two fragments, termed p33 and p55. Open arrows indicate the most common site at which wild-type VacA from *H. pylori* 60190 undergoes spontaneous cleavage and the resulting p33 and p55 fragments. Closed arrows indicate the enterokinase cleavage site introduced with the FLAG-tag epitope and the FLAGp33 and p55 fragments generated after enterokinase cleavage. The amino acid numbering system used in this figure is based on designating the first amino acid (alanine) of the mature secreted VacA toxin of strain 60190 as amino acid 1.

This enterokinase cleavage site provided a mechanism by which the 88 kDa VacA protein could be specifically cleaved into fragments similar to the spontaneously arising 33 kDa and 55 kDa VacA fragments. The FLAG-VacA toxin was purified from *H. pylori* VM088 culture supernatant and underwent spontaneous partial degradation into two forms of the p33 and p55 VacA fragments (Fig. 10A and B), which is consistent with previous observations suggesting that proteolysis can occur at multiple sites. As expected, enterokinase treatment resulted in the complete cleavage of the full-length FLAG-VacA into p55 and FLAG-tagged p33 (FLAGp33) fragments as seen by immunoblot analysis using an anti-VacA serum or an anti-FLAG monoclonal antibody (Fig. 10A and B).
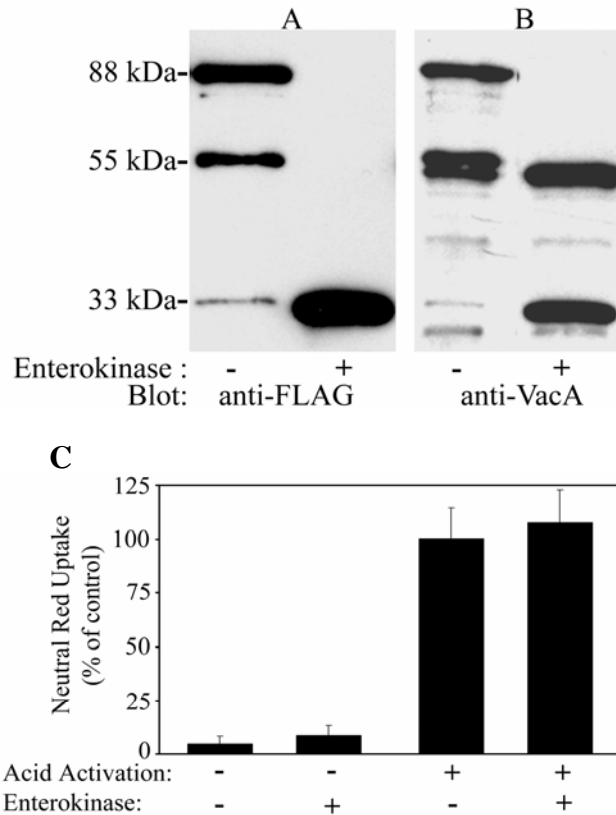
Figure 10. Characterization of the FLAG-VacA. Purified VacA from *H. pylori* VM088 (10 μg) was either treated with enterokinase (1 Unit) or mock treated. Toxins (100 ng) were then electrophoresed on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and reacted with anti-FLAG M2 monoclonal antibody (Sigma) (A). The blot was then stripped and reacted with polyclonal anti-VacA serum (B). Enterokinase treatment induced complete cleavage of full-length FLAG-VacA into ~33 and ~55 kDa fragments. In preparations of FLAG-VacA not treated with enterokinase, the FLAG-tag epitope is detected predominantly in full-length 88 kDa VacA and in the p55 fragment (Figure 6A lane 1). Only the ~33 kDa fragment retained the FLAG-tag epitope after enterokinase treatment (Panel A lane 2). C) Identical aliquots of purified FLAG-VacA were either treated with enterokinase as described above or left untreated. Toxins (5 μg/ml) were then either acid activated (pH 3) or left untreated and added to tissue culture medium overlying HeLa cells. Vacuolating activity was quantified using a neutral red uptake assay. Enterokinase-treated FLAG-VacA and untreated FLAG-VacA did not differ significantly in vacuolating cytotoxin activity. Furthermore, both enterokinase-treated and untreated FLAG-VacA required acid activation in order to induce vacuolating activity. Results represent the mean $\pm$ S.D. from triplicate samples and are expressed as a percent of neutral red uptake induced by full-length acid-activated FLAG-VacA.

Proteolytically cleaved VacA exhibits vacuolating activity
similar to that of full-length VacA

We next compared the cytotoxic activities of intact FLAG-VacA and FLAG-VacA that had been proteolytically cleaved into p55 and FLAGp33 fragments. Purified oligomeric FLAG-VacA was either treated with enterokinase or mock treated as described in In the Methods section. Proteins were then acid activated (a procedure used to enhance vacuolating activity) (21, 53) and added to the neutral-pH medium overlying HeLa cells. The vacuolating activities of intact FLAG-VacA and proteolytically cleaved FLAG-VacA were indistinguishable as determined a neutral red uptake assay (Fig. 10C).

VacA purified from wild-type *H. pylori* strain 60190 exhibits very little vacuolating toxic activity unless it is acid- or alkaline-activated prior to contact with cells (21, 53, 108). This acid- or alkaline activation induces the disassembly of the VacA oligomer into monomeric proteins, suggesting perhaps that VacA needs to be in a monomeric form to bind to mammalian cells and to form active channels. We hypothesized that proteolytically cleaved VacA might exhibit vacuolating toxic activity even in the absence of acid-activation. However, neither intact nor proteolytically cleaved FLAG-VacA exhibited detectable vacuolating activity in the absence of acid activation, as determined by a neutral-red uptake assay (Fig. 10C).

Proteolytically cleaved VacA forms oligomeric structures similar to full-length VacA

The secreted FLAG-VacA assembles into large water-soluble oligomeric structures, in a manner similar to wild-type VacA (Fig. 11A) (16). To determine whether the p33 and p55 domains remained physically associated following proteolytic cleavage

of FLAG-VacA, enterokinase-treated FLAG-VacA was fractionated by gel filtration chromatography (16), and its elution pattern monitored by immunoblot analysis with an anti-VacA serum. Proteolytically cleaved FLAG-VacA and intact FLAG-VacA were found to elute in the same high molecular mass fractions (Fig. 11; compare panels A and B), indicating that VacA fragments (i.e. FLAGp33 and p55) remain physically associated within an oligomeric structure.
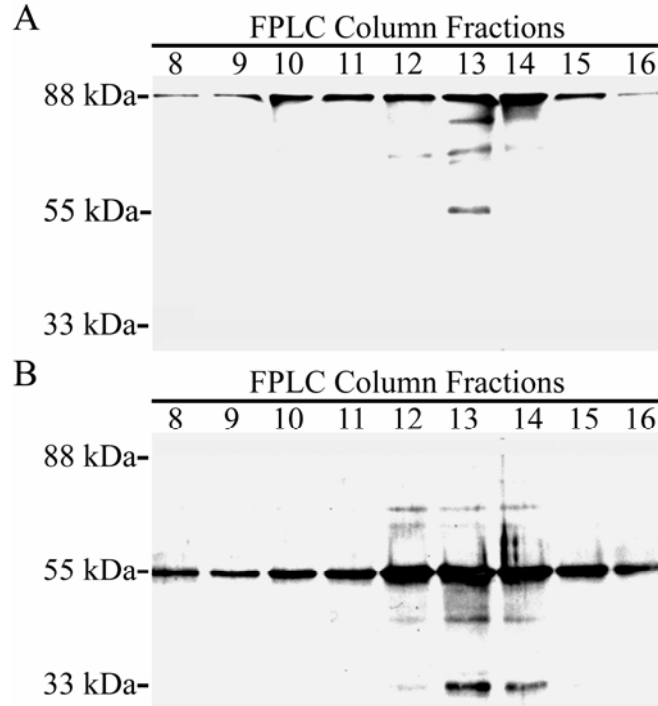
Figure 11. Oligomeric state of full-length FLAG-VacA and cleaved FLAG-VacA. Concentrated broth culture supernatant from *Helicobacter pylori* strain VM088 (A) and 40 μg of purified FLAG-VacA treated with enterokinase as described in In the Methods section (B), were applied to a Superose 6 HR FPLC column (16). Forty fractions of 2 ml each were collected, with fraction 1 corresponding to the void volume. Samples (40 μl) were then electrophoresed on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and immunoblotted with anti-VacA serum. Full-length VacA as well as enterokinase-cleaved VacA eluted in the same fractions with an elution peak at fraction 13, corresponding to a molecular mass of about 1,000 kDa. The p55 fragments are detected more prominently than p33 fragments in panel B due to the relatively weaker reactivity of the anti-VacA serum against the p33 fragment.

Discussion

Although many lines of evidence indicate that VacA can form oligomeric structures (1, 16, 46, 100), thus far there has been relatively little investigation of the specific VacA-VacA interactions that are required for its assembly into higher order structures. In this study we investigated the role of p33 and p55 interactions in VacA oligomerization. We demonstrated that only heterotypic interactions between p33 and p55 were detected using the yeast two-hybrid system (Fig. 6). The lack of detectable p33/p33 and p55/p55 homotypic interactions was not due to the lack of expression of these fusion proteins in the yeast, since all four fusion proteins were able to interact when tested for p33/p55 interactions (Fig. 6). Importantly, the failure to detect p33/p33 or p55/p55 interactions in the yeast two-hybrid system does not exclude the possibility that such interactions might occur in other environments. Previously it has been reported that a modified p55 fragment secreted by *H. pylori* can form homodimers but not large oligomeric structures (76). However, the VacA protein analyzed in that study contained at least 27 residues of the p33 domain in addition to the p55 fragment (76). In addition, another previous study provided evidence that an amino-terminal hydrophobic portion of the p33 fragment could undergo transmembrane protein homodimerization within *E. coli* membranes (49). The inability to detect p33/p33 interactions with the yeast two-hybrid system is consistent with a model in which p33/p33 interactions occur only within membranes (49, 52).

In an effort to map putative interacting regions within p33 and p55, we used the yeast two-hybrid system to analyze a series of mutant proteins containing internal

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deletions, as well as truncated VacA proteins Fig. 7 and 8) (100). The yeast two-hybrid data suggest that amino acids 28-196 within the p33 domain and 313-478 within the p55 domain contribute to the p33/p55 interaction. Only one of the p33 deletion mutants (Δ6-27p33) was able to interact with wild-type p55. VacA amino acids 6-27 comprise a hydrophobic region of VacA that inserts into lipid membranes and is required for VacA cytotoxic and membrane anion-channel activity, but not for VacA oligomerization (100).

By constructing an enterokinase sensitive VacA toxin (Fig. 10), which can be efficiently cleaved into p33 and p55 domains, we were able to study the p33/p55 interactions that occur within the VacA oligomer produced by *H. pylori*. Our results clearly indicate that p33 and p55 fragments remain physically associated after proteolytic cleavage of VacA (Fig. 11). Furthermore, our results also demonstrate that the vacuolating activity of intact FLAG-VacA and fully proteolytically cleaved FLAG-VacA is indistinguishable (Fig. 10C). A likely explanation for the intact activity of proteolytically cleaved VacA is that the fragments remain associated and do not undergo any extensive adverse conformational changes (unfolding) following proteolytic cleavage.

Two lines of evidence presented in the current study suggest that the p33/p55 interactions play an important role in VacA oligomer assembly. First, the intact oligomeric structure of proteolytically-cleaved VacA indicates that p33/p55 interactions occur within VacA oligomers (Fig. 11B). Second, the properties of mutant p33 fragments in the yeast two-hybrid system correlate perfectly with the capacity of secreted *H. pylori* mutant proteins to form oligomeric structures (Fig. 7). Together, these data suggest that mutations disrupting the p33/p55 interaction also disrupt the formation of

large VacA oligomeric structures. It is possible that there may be two different types of interactions: (i) intramolecular interactions between the p33 and p55 domains of an individual VacA monomer that might be important for proper folding of the VacA monomers within the oligomeric structures and (ii) intermolecular interaction between p33 and p55 domains of different 88 kDa molecules to form the larger oligomeric structures. Currently, we are not able to differentiate between these two interactions.

VacA toxins that fail to oligomerize consistently lack vacuolating cytotoxic activity, suggesting that oligomerization is important for VacA activity (Fig. 7A) (100). This hypothesis is supported by the observation that neither p33 nor p55 are able to exhibit vacuolating cytotoxic activity in a transient transfection assay when expressed individually within cells (20, 111, 113). In contrast, when both domains are co-expressed within cells, they interact and exhibit vacuolating cytotoxic activity (111, 113). Thus, the data presented in the current study, as well as data obtained using other systems, indicate that p33/p55 interactions are essential for VacA assembly into oligomeric structures and also for vacuolating cytotoxic activity.

CHAPTER III

FUNCTIONAL PROPERTIES OF THE P33 AND P55 DOMAINS OF THE *HELICOBACTER PYLORI* VACUOLATING CYTOTOXIN

Introduction

Helicobacter pylori secretes a vacuolating cytotoxin (VacA) that induces extensive vacuolating activity on mammalian cells (15, 43). In order for VacA to be cytotoxic, it must bind to the plasma membrane and then be internalized by host cells (53). The mature secreted 88 kDa VacA toxin can undergo proteolytic degradation into two fragments, p33 and p55, which are considered to be two VacA domains (90, 94). However, detailed analyses of the functional roles of p33 and p55 domains in VacA activities have not yet been performed. One of the major constraints during the past decade in efforts to investigate VacA structure-function relationships has been the lack of a recombinant expression system to produce active VacA proteins. Recently, McClain and Cover described a methodology to express functionally active full-length VacA proteins using an *E. coli* expression system (51). In the current study, this recombinant methodology was used to produce recombinant p33 and p55 VacA proteins. These recombinant VacA proteins were then used to investigate the role of p33 and p55 domains in several processes relevant to VacA intoxication.

Methods

Bacterial, media, and growth condition: *Escherichia coli* DH5α was used for plasmid propagation and was grown in Luria-Bertani (LB) broth or LB agar at 37 °C. For expression of the recombinant proteins, expression plasmids were transformed into *E. coli* strain JM109-DE3 (Stratagene), which encodes an isopropyl-ß-D-thiogalactopyranoside (IPTG)-inducible copy of the T7-RNA polymerase gene from bacteriophage T7. Transformants were grown in Terrific broth (TB; Invitrogen) supplemented with 25 µg of kanamycin/ml (TB-KAN).

Construction of VacA expression plasmids: A *vacA* sequence encoding the VacA p33 domain (amino acids 1-312 of the mature secreted toxin) with a 6-His tag at the C-terminus was PCR-amplified from the pMM592 plasmid (51). The PCR product was digested with SpeI and SalI and ligated into XbaI- and SalI-digested pET-41b (conferring kanamycin resistance; Novagen) to create the plasmid p145 (p33His). We also generated a plasmid that encoded a VacA p33 domain with two tags (c-Myc and 6-His), each at the carboxyl-terminus of the protein (p33Myc-His).

A *vacA* sequence encoding the p55 domain (amino acid 312-821) with a c-Myc tag (p55Myc) at the amino-terminus was PCR-amplified from *H. pylori* VT320 genomic DNA (50). Also, a *vacA* sequence encoding the p55 domain with a 6-His tag at the N-terminus was PCR-amplified from the pMM592 plasmid (51). PCR products were digested and cloned into pET-41b as described above. Also, 6-His tagged-p55 fragments encoding amino acids 312-780, 312-700, 312-550, and 312-478 were PCR-amplified

from the pMM592 plasmid. The PCR products were digested and cloned into pET-41b as described above.

Expression of recombinant VacA proteins: VacA expression plasmids were transformed into the *E. coli* expression strain JM109-DE3, and transformants were then inoculated into TB-KAN and grown at 37 °C overnight with shaking (51). Cultures were then diluted 1:100 into TB-KAN, and incubated at 37°C until they reached an optical density (OD_{600}) of 0.5. Cultures were then induced with a final IPTG concentration of 0.5 mM and incubated at 25 °C for 16-18 h (p55 proteins and full-length VacA proteins) or at 37°C for 2 h (p33 proteins). *E. coli* soluble extracts were generated as described by McClain *et at* (51). Briefly, 50 ml of IPTG-induced cultures were pelleted, washed in 0.9% NaCl, and resuspended in a solution (1 ml) that contained 10 mM Tris (pH 7.5), 100 mM NaCl, 1 mM EDTA, protease inhibitors (Complete Mini; Roche), and 20,000 U of ReadyLyse lysozyme (Epicentre) per ml. Bacteria cells were incubated on ice for 30 min with periodic mixing, after which a solution (3 ml) containing 50 mM Tris (pH 8.0), 2.67 mM $MgCl_2$, and 67 U of Benzonase-nuclease (Novagen) per ml was added. Samples were then mixed briefly and subjected to four successive rounds of freezing (in a dry ice-methanol bath) and thawing at 37°C, and the insoluble debris was pelleted. The *E. coli* soluble extracts containing the VacA proteins were collected and stored at -20 °C until use.

Immunoblot analysis of recombinant VacA: *E. coli* soluble extracts containing the VacA proteins were generated as described above and the proteins were separated by

SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted using a polyclonal anti-His antibody (Santa Cruz), a monoclonal anti-c-Myc (9E10) antibody, or a polyclonal anti-VacA antibody (958), and secondary antibodies conjugated with horseradish peroxidase (Bio-Rad). Signals were generated by the enhanced chemiluminescence reaction (Roche) and detected using x-ray film. For subsequent experiments, *E. coli* soluble extracts were normalized to contain similar amounts of recombinant VacA proteins based on densitometry analysis of the x-ray films.

Cell culture and vacuolating assays: HeLa cells were grown in minimal essential medium (modified Eagle's medium containing Earle's salts; MEM) supplemented with 10% fetal bovine serum. For vacuolating assays, HeLa cells were seeded into 96-well plates 24 h prior to each experiment. To test the *E. coli* soluble extracts for vacuolating activity, aliquots of each extract were normalized to contain similar amounts of recombinant VacA protein by immunoblotting with an anti-His antibody as described above, and then samples were added to the medium overlying HeLa cells (supplemented with 50 μg per ml of gentamicin and 10mM ammonium chloride) for 1 h (15). For the p33/p55 complementation assays, *E. coli* soluble extracts were mixed and incubated for 1 h at room temperature prior to addition to the medium overlying HeLa cells. For experiments in which p33 and p55 samples were added sequentially to cells, lysate containing the first VacA protein was added to the cells and incubated for 1h. After incubation, the cells were washed two times to remove unbound proteins and lysate containing the second VacA protein was then added for an additional 1h. Cells were then washed two more times and the culture media overlying cells was replaced with fresh

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culture media and incubated for 4-6 h at 37 °C. Purified VacA from *H. pylori* culture supernatants was acid-activated by adjusting the VacA protein to pH 3 by the addition of 250 mM hydrochloric acid before they were added to cell culture wells (21), whereas *E. coli* lysates were not acid-activated. In all vacuolating assays, the medium overlaying HeLa cells was supplemented with 10mM ammonium chloride and gentamicin. After incubation, cells vacuolation was examined by inverted light microscopy and quantified by a neutral red uptake assay as described previously. Neutral red uptake data are presented as OD_{540} values (means $\pm$ S.D.). Statistical analysis was made by ANOVA and Dunnett's post-hoc test.

Immunoprecipitation of VacA complexes: *E. coli* soluble extracts, normalized to contain similar amounts of recombinant VacA proteins, were mixed and incubated for 1 h as described above. VacA complexes were then immunoprecipitated with an anti-c-Myc monoclonal antibody (2 μg of clone 9E10) and protein G coated-beads. Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with an anti-His (Santa Cruz) antibody or anti-c-Myc monoclonal antibody, and secondary antibodies conjugated with horseradish peroxidase (Bio-Rad). Signals were generated by the enhanced chemiluminescence reaction (Roche) and detected using x-ray film.

Analysis of VacA binding and internalization into mammalian cells: Normalized *E. coli* soluble extracts containing single recombinant VacA proteins or mixtures of the recombinant VacA proteins were incubated for 1h as described above, and samples were

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then added to HeLa cells grown on cover glasses in 6 well plates for 1h at 37 °C. VacA interactions with mammalian cells were then analyzed by indirect immunofluorescence. Briefly, cells were washed with tris-buffered saline (TBS) and fixed with 3.7% formaldehyde. Fixed cells were then incubated with an anti-c-Myc antibody (1:500) or with an anti-VacA polyclonal antibody (958) that recognizes the p55 domain, for 1 h at room temperature (81). Cells were then washed and incubated with a Cy3-conjugated secondary antibody (1:500) for 1 h at room temperature. To analyze VacA internalization into host cells, *E. coli* soluble extracts containing single recombinant VacA proteins or mixtures of recombinant VacA proteins were treated as described above and then incubated with HeLa cells for 1h at 37 °C (44). Afterward, the medium was changed and cells were incubated for 12-16 h at 37 °C. VacA intoxicated cells were then washed with TBS, fixed with 3.7% formaldehyde, and permeabilized with 100% methanol for 30 minutes at -20 °C. Cells were then incubated with an anti-VacA polyclonal or an anti-c-Myc antibody followed by a Cy3-conjugated secondary antibody. After immunolabeling, cover glasses were washed with phosphate-buffered saline, mounted on slides with Aqua-Polymount (Polysciences, Warrington, PA), and viewed with a LSM 510 confocal laser scanning inverted microscope (Carl Zeiss, Jena, Germany) (44).

For immunoblot analysis of VacA interaction with cells, HeLa cells were seeded into a 96-well plate and intoxicated with normalized *E. coli* soluble extracts as described above. Cells were then washed three times with TBS and then lysed directly in the wells of the 96-well plate by adding SDS-loading buffer. Proteins were then analyzed by immunoblotting as described above.

Results

Expression of recombinant p33 and p55 VacA domains

Purified 88 kDa VacA protein from *H. pylori* culture supernatant can undergo proteolysis into two fragments designated as p33 and p55 VacA domains (Fig. 12A) (90, 94). Currently, there are only several systems that can be used to study p33 and p55 structure and function. These systems include transient transfection assays (20, 107), yeast two-hybrid analysis (94) and analysis of purified toxins from *H. pylori* broth culture supernatants (15). Among these systems, only analysis of proteins produced by *H. pylori* allows for the study of VacA-cell interactions, but structure-function analysis of VacA using the *H. pylori* system is cumbersome and in some cases impossible. To enable further functional studies of the p33 and p55 domains, we adapted the VacA expression methodology reported by McClain and Cover (i.e. a methodology that allows for the expression of functionally active recombinant VacA proteins) to produce recombinant p33 and p55 VacA proteins (51). The *vacA* sequences encoding the p33 and p55 were cloned into the pET-41b *E. coli* expression plasmid. The plasmids were then transformed into an *E. coli* expression strain, recombinant proteins were expressed, and soluble extracts containing the recombinant p33 and p55 VacA proteins were generated as described in the Methods section (Fig. 12B).
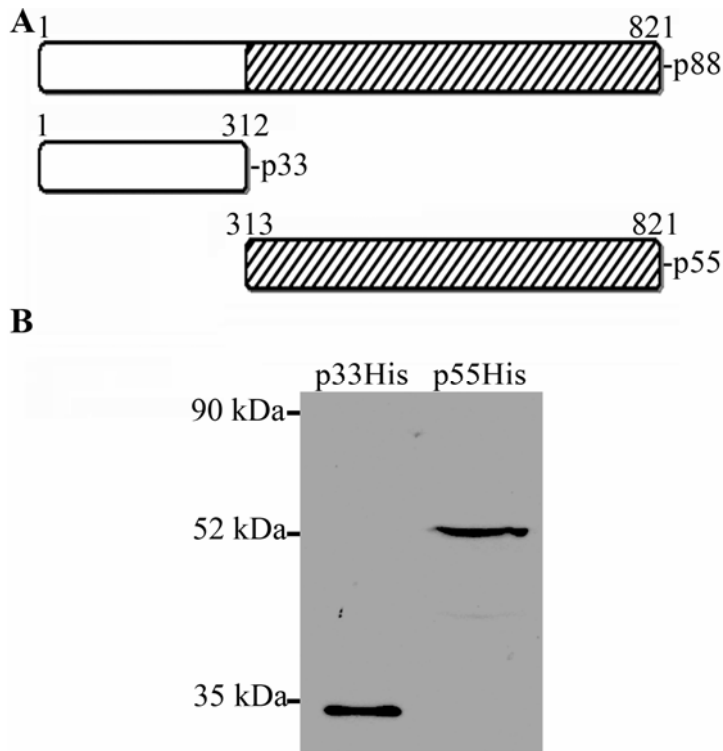
Figure 12. Expression of the p33 and p55 VacA domains in *E. coli*. *A*) Diagram of the mature secreted ~88 kDa wild-type VacA protein (p88) and the ~33 kDa (p33) and ~55 kDa (p55) VacA domains. The p33 domain is comprised of amino acids 1-312 and the p55 domain is comprised of amino acids 313-821. The VacA amino acid numbering system used in this figure is based on designating the first amino acid (alanine) of the mature secreted VacA toxin of *Helicobacter pylori* strain 60190 as amino acid 1. *B*) Plasmids encoding His-tagged VacA p33 or p55 domains were transformed into the *E. coli* expression strain (JM109-DE3). Proteins were expressed and soluble extracts containing the VacA proteins were generated as described in the Methods section. Soluble proteins were electrophoresed on a 12% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and immunoblotted with an anti-His antibody (Santa Cruz).

p33 and p55 VacA domains complement each other for vacuolating activity

Previously, it has been shown that *E. coli* soluble extract containing the full-length wild-type recombinant VacA exhibits vacuolating activity when added to mammalian cells (51). Therefore, we used *E. coli* soluble extract containing the p33 and p55 proteins to investigate whether these VacA proteins were also capable of inducing vacuolating activity when added to mammalian cells. No detectable vacuolating activity was observed when HeLa cells were intoxicated with lysates containing p33 or p55 proteins (Fig. 13). We then investigated whether the p33 and p55 proteins could complement each other for vacuolating activity. Accordingly, *E. coli* soluble extracts containing the p33 and p55 proteins were mixed and then added to HeLa cells. When mixed together the p33 and p55 VacA proteins exhibited extensive cell vacuolation, as evidenced by the increase in neutral red uptake (Fig. 13A). The vacuolating activity exhibited by the p33/p55 mixture was comparable to the activity exhibited by 10 μg/ml of purified acid-activated VacA from *H. pylori* broth culture supernatant. Similar extensive vacuolating activity was also observed when a gastric cell line (i.e. AZ-521), was intoxicated with the p33/p55 mixture instead of HeLa cells (Fig 13B). As seen with purified VacA, the vacuolating activity exhibited by the mixture of the recombinant p33 and p55 VacA domains was dose-dependent (Fig 13C). These results indicate that recombinant p33 and p55 VacA domains are capable of complementing each other, resulting in vacuolating cytotoxic activity.
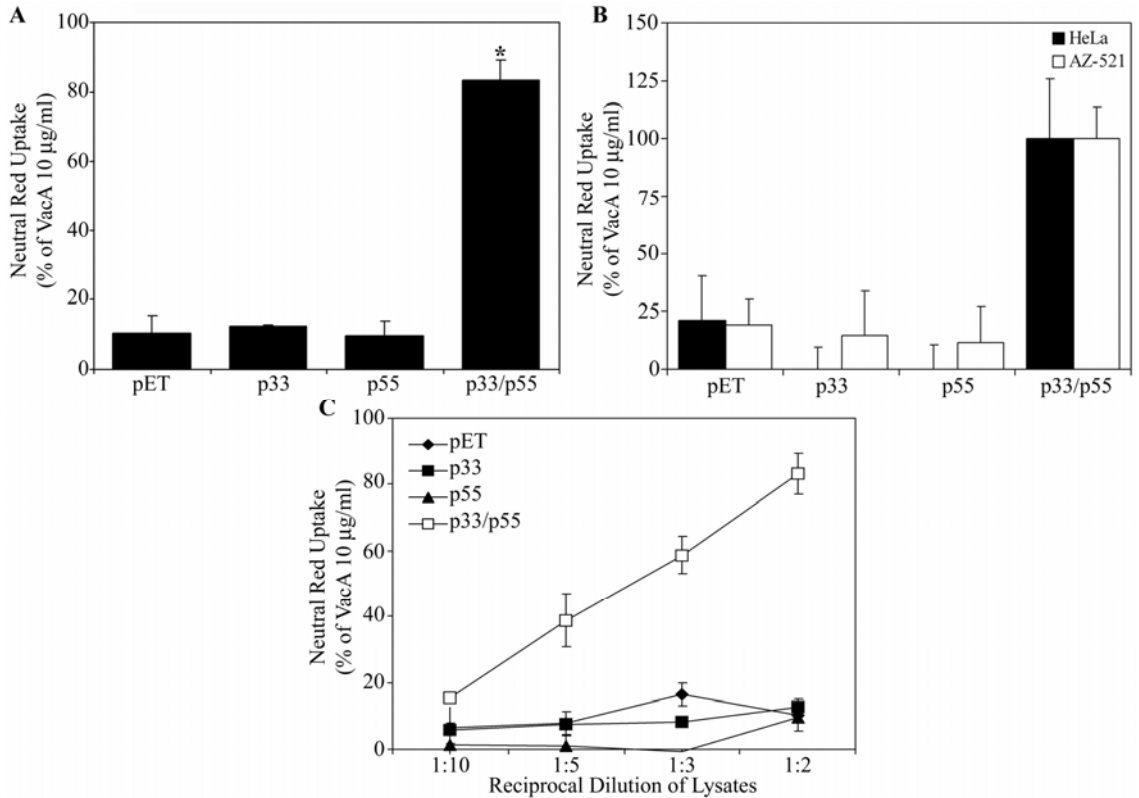
Figure 13. The recombinant p33 and p55 VacA domains exhibit vacuolating activity when combined, but not when added alone to mammalian cells. *A*) *E. coli* soluble extracts containing single recombinant p33His and p55His VacA proteins, negative control lysate without VacA protein (pET), or a mixture of extracts containing the p33 and p55 domains were added to HeLa cells as described in the Methods section. Lysates were normalized by densitometry analysis of the immunoblot x-ray films, such that the relative amounts of p33 and p55 domains were approximately equivalent. Vacuolating activity was measured by a neutral red uptake assay. Results represent the mean ± standard deviation from triplicate samples, expressed as a percent of neutral red uptake induced by 10 μg/ml of full-length acid-activated VacA toxin purified from *H. pylori* culture supernatant. Comparisons between each sample and the pET control were made by ANOVA and Dunnett's post-hoc test. Asterisks denote results significantly different from control ($p<0.05$). These data suggest that both the p33 and p55 domains are required for VacA cytotoxic activity. *B*) HeLa and AZ-521 cells were intoxicated with the p33 and p55 proteins as described in panel A. Vacuolating activity was measured by a neutral red uptake assay, and results represent the mean ± standard deviation from triplicate samples of two independent experiments, expressed as a percent of neutral red uptake induced by the p33/p55 mixture. These data suggest that p33/p55 mixtures exhibit similar vacuolating activity in gastric cell lines. *C*) Dilutions of the normalized *E. coli* soluble extracts containing the indicated proteins as in panle A were added to HeLa cells as described above. Vacuolating activity was measured by a neutral red uptake assay. Results represent the mean ± standard deviation from triplicate samples, expressed as a percent of neutral red uptake induced by 10 μg/ml of full-length acid-activated VacA toxin purified from *H. pylori* culture supernatant.

The C-terminal region of p55 is important for maximum vacuolating cytotoxic activity

In an effort to map the minimal p55 protein capable of complementing the p33 domain for vacuolating activity, we generated a series of p55 proteins truncated at the C-terminus. The truncated p55 proteins were expressed in *E. coli* (Fig. 14A) and then tested for their ability to exhibit vacuolating activity when mixed with the p33 VacA domain. A p55 truncated protein containing amino acids 312-780 complemented the p33 domain, resulting in high levels of vacuolating activity (Fig. 14B). In contrast, a p55-truncated protein containing amino acids 312-700 was impaired in its capacity to complement p33 for vacuolating activity, and no detectable cytotoxic activity was observed when p55 truncated proteins containing amino acids 312-550, or 312-478 were mixed with p33 (Fig. 14B). We also performed experiments designed to test the effect of these C-terminal truncations on the activity of full-length recombinant VacA. Similar to the results obtained with the truncated p55 proteins (Fig. 14B), a truncated VacA protein containing amino acids 1-780 exhibited extensive vacuolating activity, a VacA mutant protein containing amino acids 1-700 was impaired in its vacuolating activity, and a mutant protein containing amino acids 1-478 was completely inactive (Fig. 14C). These data indicate that the p55 domain retains detectable functional activity when small segments of the C-terminus are deleted, but that activity is progressively lost with larger C-terminal deletions.
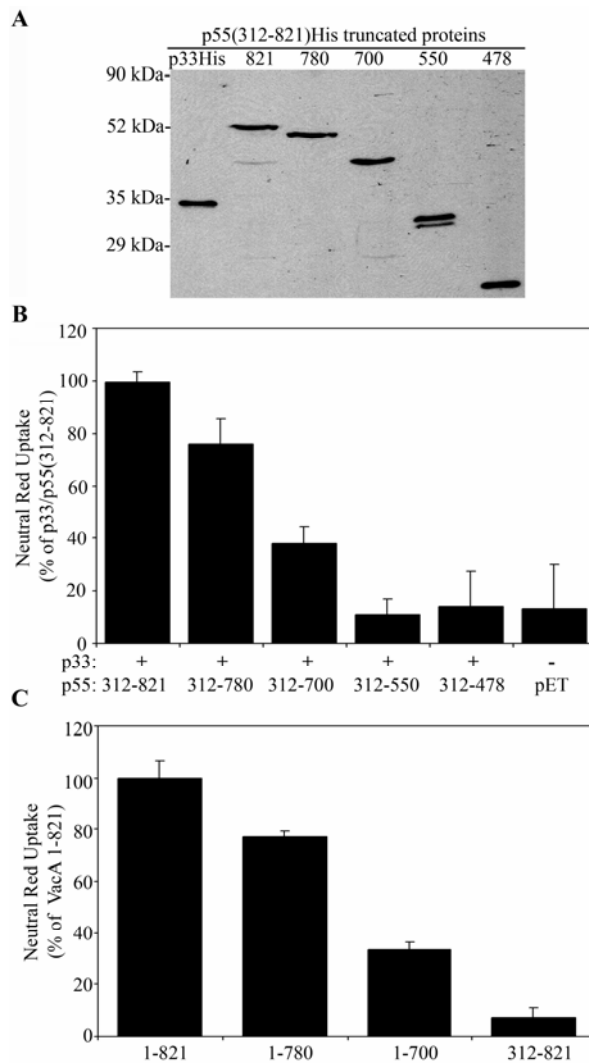
Figure 14. Mapping the minimum portion of p55 required for vacuolating activity. *A*) Plasmids encoding p33His, wild-type p55His (amino acids 312-821), and C-terminal truncated versions of the p55His protein were transformed into the *E. coli* expression strain (JM109-DE3). Proteins were expressed and soluble extracts containing the p55 proteins were generated as described in the Methods section. Soluble proteins were electrophoresed on a 12% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and immunoblotted with an anti-His antibody (Santa Cruz). *B*) *E. coli* soluble extracts containing a mixture of p33 with p55-(312-821), p55-(312-780), p55-(312-700), p55-(312-550), or negative control (pET) were added to HeLa cells. Normalized input proteins used in this experiment are shown in panel A. Vacuolating activity was measured by a neutral red uptake assay and the results represent the mean $\pm$ standard deviation from triplicate samples, expressed as a percent of neutral red uptake induced by p33/p55(312-821) samples. *C*) *E. coli* soluble extracts containing the full-length recombinant VacA (1-821), or the truncated VacA recombinant proteins (1-780, 1-700, and 312-821) were added to HeLa cells as described above. Vacuolating activity was measured by a neutral red uptake assay. Results represent the mean $\pm$ standard deviation from triplicate samples, expressed as a percent of neutral red uptake induced by the recombinant 1-821 VacA protein.

p33 and p55 domains form oligomeric complexes

We hypothesized that the ability of the p33 and p55 proteins to complement each other for vacuolating activity might require the formation of protein complexes comprised of these two proteins. To test whether the VacA domains could physically interact, we prepared *E. coli* soluble extracts containing the p33 and p55 proteins tagged with His- and/or c-Myc-tags and then performed a series of immunoprecipitation experiments. In these experiments, only p33/p55 interactions (Fig. 15A; lanes 4 and 7), but not p55/p55 (Fig. 15A; lane 5) or p33/p33 (Fig. 15A; lane 6) interactions were detected. These data indicate that the recombinant p33 and p55 proteins are capable of interacting in solution to form p33/p55 protein-complexes.

To determine whether the p33 and p55 domains form only simple binary complexes or also higher ordered complexes in solution, we mixed different tagged versions of the p33 and p55 domains (p33His, p55His, and p33Myc-His) and then immunoprecipitated the protein complexes using an anti-c-Myc antibody. When samples containing the p33His, p55His, and p33Myc-His proteins were mixed, they formed protein complexes comprised of all three proteins (p33Myc-His, p33His and p55His; Fig. 15B, lane 3). The ability to form these p33Myc-His/p33His/p55His complexes was dependent on the p33/p55 interaction (Fig. 15B; lane 2), since p33His and p33Myc-His failed to interact when mixed in the absence of p55 (Fig. 15B; lane 1). These data indicate that p33/p55 protein complex can potentially be composed of at least three independent subunits. However, we were unable to detect the assembly of the p33 and p55 domains into large 1,000 kDa complexes similar to those formed by the 88 kDa

secreted VacA proteins present in *H. pylori* broth culture supernatant, based on analysis involving gel filtration chromatography (16).

Interactions of p33 and p55 domains with full-length 88 kDa VacA

We next investigated whether the p33 and p55 proteins could form mixed oligomeric complexes with wild-type 88 kDa VacA protein purified from *H. pylori* culture supernatants. To this end, *E. coli* soluble extracts containing p33, p55, or the p33/p55 mixture were mixed with a full-length 88 kDa c-Myc tagged VacA (Myc-VacA) purified from *H. pylori* supernatant and the proteins were then immunoprecipitated with an anti-c-Myc antibody as described above. Interestingly, we were not able to detect mixed oligomer formation between the Myc-VacA and either the p33 or the p55 domains when these domains were tested independently (Fig. 15C). In contrast, when the p33 and p55 domains were mixed together with the Myc-VacA, they formed mixed complexes composed of Myc-VacA, p33 and p55 (Fig. 15C). These data provide evidence indicating that both the p33 and p55 domains play a role in the process of VacA assembly into oligomeric structures in solution.
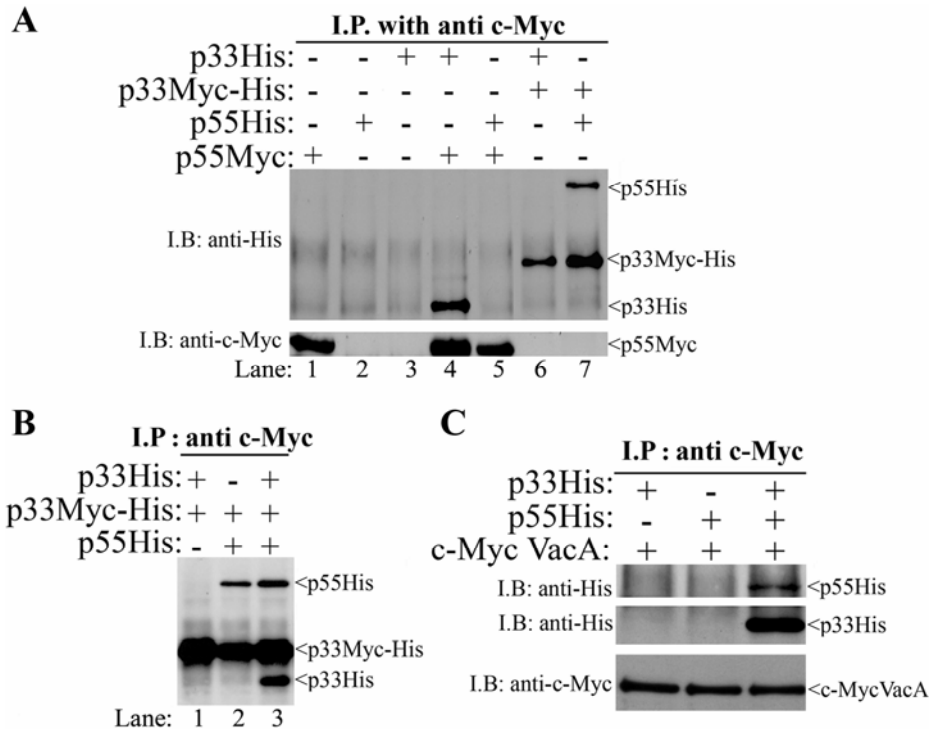
Figure 15. p33 and p55 interact in solution to form oligomeric complexes. *A*) Epitope-tagged VacA domains were expressed as described in In the Methods section. *E. coli* soluble extracts containing similar amounts of p33His, p33Myc-His, p55His, p55Myc alone or the indicated combinations were incubated with an anti-c-Myc antibody and proteins were immunoprecipitated (I.P.) as described in the Methods section. Immunoprecipitated proteins were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and immunobloted (I.B.) with an anti-His antibody (top panel) or anti-c-Myc antibody (bottom panel). These data (lanes 4 and 7) suggest that p33 and p55 interact in solution to form heterotypic p33/p55 complexes. *B*) *E. coli* soluble extracts containing similar amounts of the different p33 and p55 VacA proteins alone or the indicated combinations were incubated with an anti-c-Myc antibody and proteins were immunoprecipitated as described in panel A. Immunoprecipitated proteins were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and immunobloted with an anti-His antibody. These data suggest that p33 and p55 can interact in solution to form p33/p55 complexes that are composed of at least three independent VacA domains. *C*) *E. coli* soluble extracts containing the indicated recombinant VacA proteins were mixed with acid-activated full-length c-Myc-tagged VacA protein (Myc-VacA) purified from *H. pylori* culture supernatant (2 μg) as indicated. Mixtures were then incubated with an anti-c-Myc antibody and proteins were immunoprecipitated as described in panel A. Immunoprecipitated proteins were electrophoresed on a 12% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and immunobloted with an anti-His antibody (top panels) or anti-c-Myc antibody (bottom panel). These data suggest that the p33 and p55 domains can form mixed-oligomeric complexes with full-length 88 kDa VacA only when both domains are present.

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The p33 and p55 VacA domains interact with mammalian cell membranes

In the next series of experiments, we investigated the functional roles of the p33 and p55 domains in the process whereby VacA interacts with mammalian cells. As a first approach, interaction of the p33 and p55 domains with mammalian cells was investigated by immunoblot analysis. When added individually to cells for 1 h at 4°C, both the p33 and p55 domains were able to interact with the surface of HeLa cells (Fig. 16A). However, when the p33 and p55 domains were mixed and then added to HeLa cells, the amount of p33 and p55 protein associated with cells increased dramatically compared to the amount detected when these proteins were tested individually (Fig. 16A).

As a second approach to investigate p33 and p55 interactions with mammalian cells, we used indirect immunofluorescence. The interaction of p33 with the HeLa cell surface was detected using an anti-c-Myc antibody (Fig. 16B; panels 1-4) and the interaction of p55 with the HeLa cell surface was detected using an anti-VacA polyclonal antibody (Fig. 16B; panels 5-8). As expected, full-length VacA (Myc-VacA) purified from *H. pylori* bound to the surface of HeLa cells (Fig. 16B; panels 1 and 5). The p33 (p33Myc-His) domain also bound to the surface of HeLa cells, but in contrast to the full-length Myc-VacA, the p33 domain localized in a punctate distribution on the surface of HeLa cells (Fig. 16B; panel 3). The punctate p33Myc-His distribution detected with the anti-Myc antibody was specific for this VacA protein, since no signal was detected when *E. coli* negative control lysate (pET) was used (Fig. 15B; panels 2 and 6). As shown in Fig. 16B, we were able to detect binding of the p55 domains to the surface of cells by immunoblot methodology, but we were unable to detect interaction of the recombinant p55 protein with the surface of HeLa cells using immunofluorescence assays, despite

testing two different forms of this protein (p55His or p55Myc) and multiple antibodies, including the anti-VacA polyclonal used in panel A (Fig. 16B; panel 7). We presume that the relevant epitopes are not accessible to the antibodies under the conditions of the immunofluorescence assay. Interestingly, when *E. coli* soluble extracts containing the p33 and the p55 proteins were mixed and then added to HeLa cells, both proteins were detected on the surface of HeLa cells and were localized in a continuous pattern (non-punctate), similar to full-length VacA (Fig. 16B; panels 4 and 8). Thus, binding of p55 to the cell surface was detected by immunofluorescence assay if the p33 and p55 domains were added together to cells, but not if p55 was added independently to cells. The p33 distribution on the surface of HeLa cells was punctate when added alone to cells, whereas it was continuous (non-punctate) when p33 was added to cells together with the p55 domain (Fig. 16B; panels 3 and 4). In summary, these data indicate that the p33 and p55 VacA domains can each independently interact with mammalian cell surfaces, but the binding interactions are substantially altered if the two domains are both present.
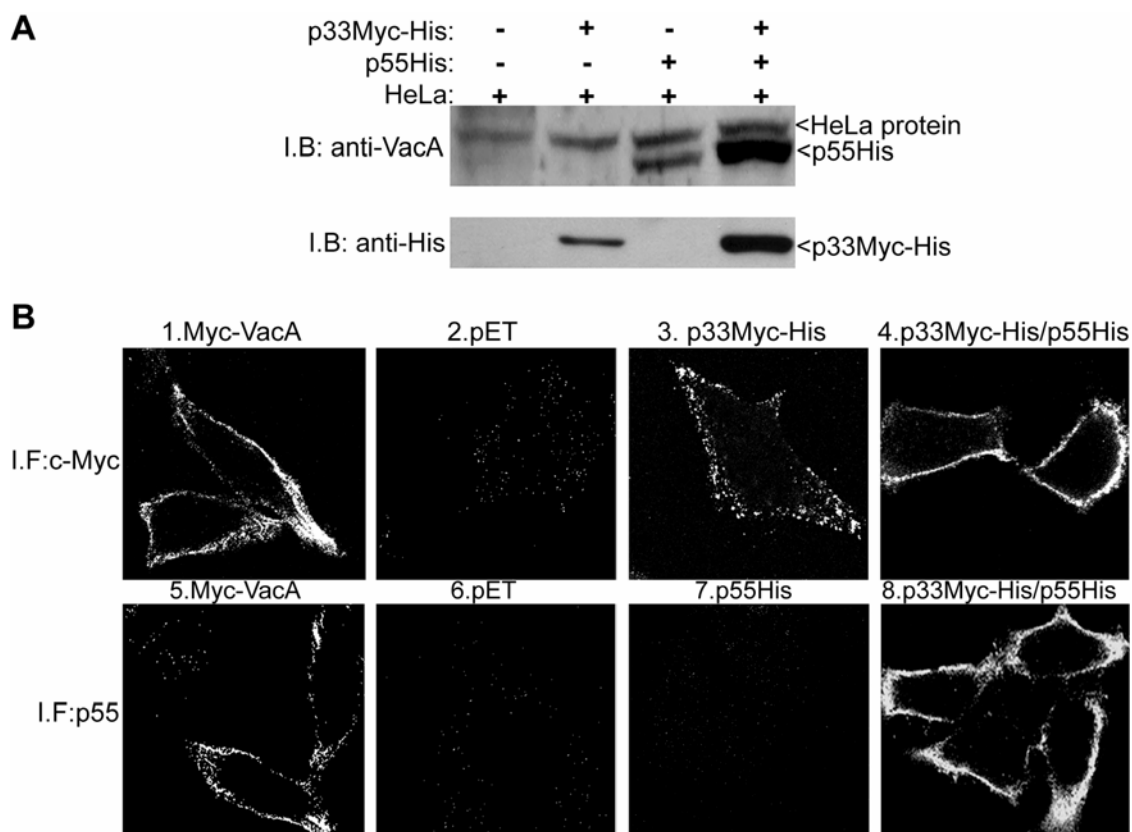
Figure 16. Both p33 and p55 VacA domains interact with mammalian cells.
A) *E. coli* soluble extracts containing similar amounts of the indicated VacA proteins
were added to HeLa cells for 1h at 4°C. The capacity of the VacA proteins to interact
with host cell membranes was assessed by immunoblot (I.B.) analysis using an anti-VacA
polyclonal antibody to detect p55His and anti-His antibody to detect p33Myc-His as
described in the Methods section. Both the p33 and p55 VacA proteins interacted with
cells when added alone, but the binding of both proteins was enhanced when the p33/p55
mixture was added to cells. *B*) HeLa cells were intoxicated for 1h at 37°C with c-Myc-
VacA (Myc-VacA; 5 μg/ml) purified from *H. pylori* culture supernatant (panels 1 and 5),
E. coli negative control lysate without VacA proteins (pET; panels 2 and 6), or with *E.
coli* soluble extracts containing p33Myc-His (panel 3), p55His (panel 7), or with the
p33Myc-His/p55His (panels 4 and 8) mixture. The capacity of the VacA proteins to
interact with the cell membrane of host cells was assessed by indirect
immunofluorescence (I.F.) using anti-c-Myc to detect the p33Myc-His protein (panels 1-
4) and anti-VacA polyclonal antibody to detect the p55 protein (panels 5-8) as described
in the Methods section.

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Intracellular localization of the p33 and p55 VacA domains

Full-length 88 kDa VacA produced by *H. pylori* binds to the plasma membrane of cells and then can be internalized into target cells via an energy-dependent and temperature-dependent process (53). Therefore, we next investigated whether the p33 and p55 proteins were internalized into mammalian cells. HeLa cells were intoxicated with either purified VacA from *H. pylori, E. coli* soluble extracts containing the p33 domain, the p55 domain, or the p33/p55 mixture. Internalized VacA proteins were visualized by indirect immunofluorescence analysis of permeabilized cells using an anti-VacA monoclonal antibody (5E4) specific for the p55 domain (Fig. 17; panels 1, 3, and 5) and an anti-c-Myc antibody to detect the p33Myc-His protein (Fig. 17; panels 2 and 4). Purified VacA was internalized into HeLa cells as shown previously (Fig. 17; panel 1). Even though the p33 and p55 domains bind to cells (Fig. 16), no internalization of the p33 or p55 proteins was detected when they were added alone to cells (Fig. 17; panels 2 and 3). In contrast, when the p33 and the p55 proteins were mixed and then added to cells, both domains were internalized and localized in a similar distribution as the full-length VacA (Fig. 17; panels 4 and 5). These data indicate that both the p33 and p55 VacA domains are required for toxin internalization into target cells.
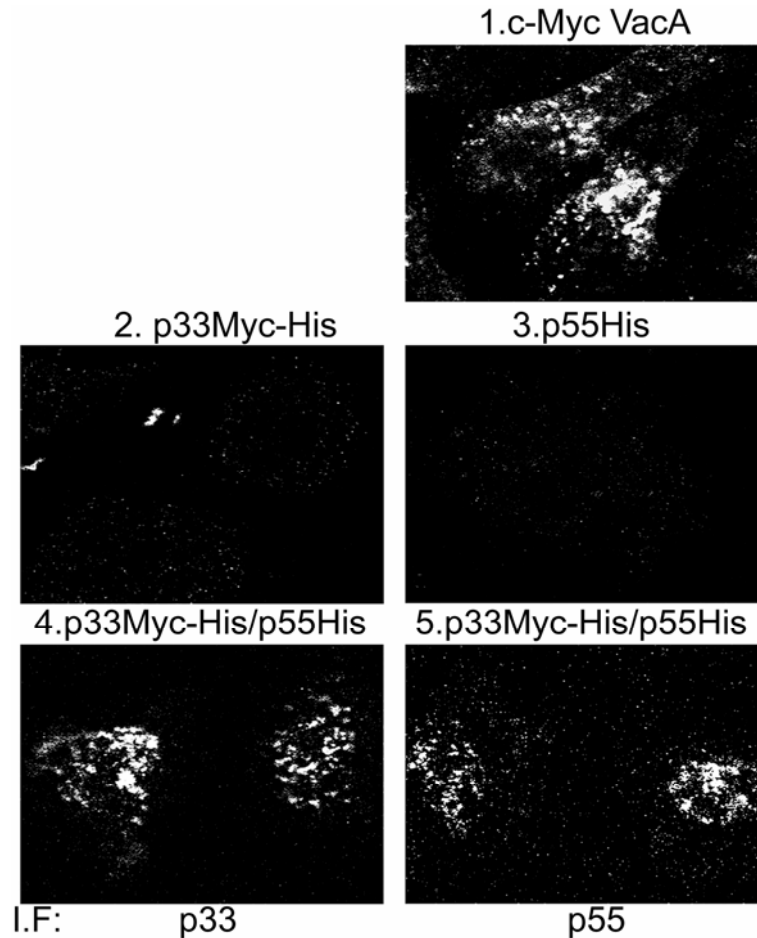
Figure 17. The p33 and p55 VacA domains are required for toxin internalization into mammalian cells. Wild-type VacA purified from *H. pylori* culture supernatant (panel 1), or *E. coli* soluble extracts containing similar amounts of p33Myc-His (panel 2), p55His (panel 3), or the p33Myc-His/p55His (panels 4 and 5) mixture were added to HeLa cells for 1.5 h at 37°C. Then the media was replaced with fresh culture media and cells were incubated for an additional 16-24 h at 37°C as described in the Methods section. The ability of VacA to enter into cells was assessed by indirect immunofluorescence (I.F.) of permeabilized cells using the 5E4 monoclonal anti-VacA antibody to detect the p55 protein (panels 1, 3, and 5) and the anti-c-Myc antibody to detect the p33Myc-His protein (panels 2 and 4), as described in the Methods section. These data suggest that the p33 and p55 VacA domains are internalized into mammalian cells when both domains are present (panels 4 and 5), but not when added individually to cells.

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Sequential addition of p55 and p33 VacA domains to cells induces cell vacuolation

To further investigate the role of p33 and p55 binding to host cells in the VacA intoxication process, we investigated whether the p33 and p55 domains could complement each other when added sequentially to HeLa cells. To this end, we intoxicated HeLa cells sequentially with *E. coli* soluble extract containing p33 followed by extract containing p55 (p33[1], p55), or p55 first and then p33 (p55[1], p33). The concentrations of p33 and p55 proteins in these experiments were approximately equivalent based on immunoblotting with an anti-His tag antibody. In each case, the media overlying cells (containing unbound VacA) was washed away prior to the addition of the second VacA protein. As shown above (Fig. 13), no vacuolating activity was observed when the pET, p33, or p55 lysates were added independently to cells, and extensive vacuolating activity was observed when the p33/p55 mixture was added to cells (Fig. 18A). When the samples were added sequentially to cells, no vacuolating activity was detected if the p33 domain was bound first followed by the addition of the p55 domain (Fig. 18A; p33[1], p55). In contrast, extensive vacuolating activity was observed if the p55 domain was bound first followed by the addition of the p33 domain (Fig. 18A; p55[1], p33).

To investigate why the (p55[1], p33) combination induced vacuolation but the (p33[1], p55) combination did not, we analyzed the interactions of sequentially added VacA proteins with the surface of host cells by immunoblot analysis. As shown above, both the p33 and p55 domains were capable of binding to the plasma membrane of HeLa cells when added independently (Fig. 18B, lanes 1 and 2), and enhanced binding to cells of both domains was detected following simultaneous addition of the p33/p55 mixtures

(Fig. 18B; lane 3). When the p55 domain was bound first followed by the p33 domain, a condition that resulted in cell vacuolation (p55[1], p33; Fig. 18A), the amount of p33 protein bound to cell membranes increased compared to when the p33 domain was added alone (Fig. 18B; bottom panel lane 2 vs. 5). Similarly, when the p33 domain was bound first followed by the p55 domain, a condition that failed to produce cell vacuolation (p33[1], p55; Fig. 18A), the amount of p55 protein bound to cell membranes increased compared to when the p55 domain was added alone (Fig. 18B; top panel lane 1 vs. 4). Similar results were obtained when different tagged p33 and p55 proteins (e.g. p33Myc-His and p55Myc) and gastric cell lines (AGS and AZ-521) instead of HeLa cells were used. These data suggest that binding of either p33 or p55 to cells enhances subsequent binding of p55 or p33, respectively.

We then investigated the distribution of sequentially bound p33 and p55 proteins on the surfaces of host cells. HeLa cells were intoxicated as described above and binding of the p33 and p55 proteins was detected by indirect immunofluorescence. The interaction of p55 with the HeLa cell surface was detected using an anti-VacA polyclonal antibody (Fig. 19A) and the interaction of p33 (p33Myc-His) with the HeLa cell surface was detected using an anti-c-Myc antibody (Fig. 19A). As shown above, when cells were intoxicated simultaneously with the p33/p55 mixture, both proteins interacted with the cells and localized in a continuous (non-punctate) pattern on the surfaces of cells (Fig. 19A, panel 1). Interestingly, when the p33 protein was added first followed by the p55 protein (p33Myc-His[1], p55His), both domains localized in a punctate distribution on the surface of the cells (Fig. 19A, panel 2). Similarly, when the p55 protein was added first followed by the p33 protein (p55His[1], p33Myc-His), both domains also localized in a

punctate distribution on the surface of the cells (Fig. 19A, panel 3). Thus, when the p33 and p55 VacA proteins are added sequentially to cells, both domains bind to the cell surface in a punctate distribution, and the pattern of binding is similar in the (p33[1], p55) and (p55[1], p33) experiments. However, these data do not provide a clear explanation for why the (p55[1], p33) combination induces cellular vacuolation but the (p33[1], p55) combination does not.
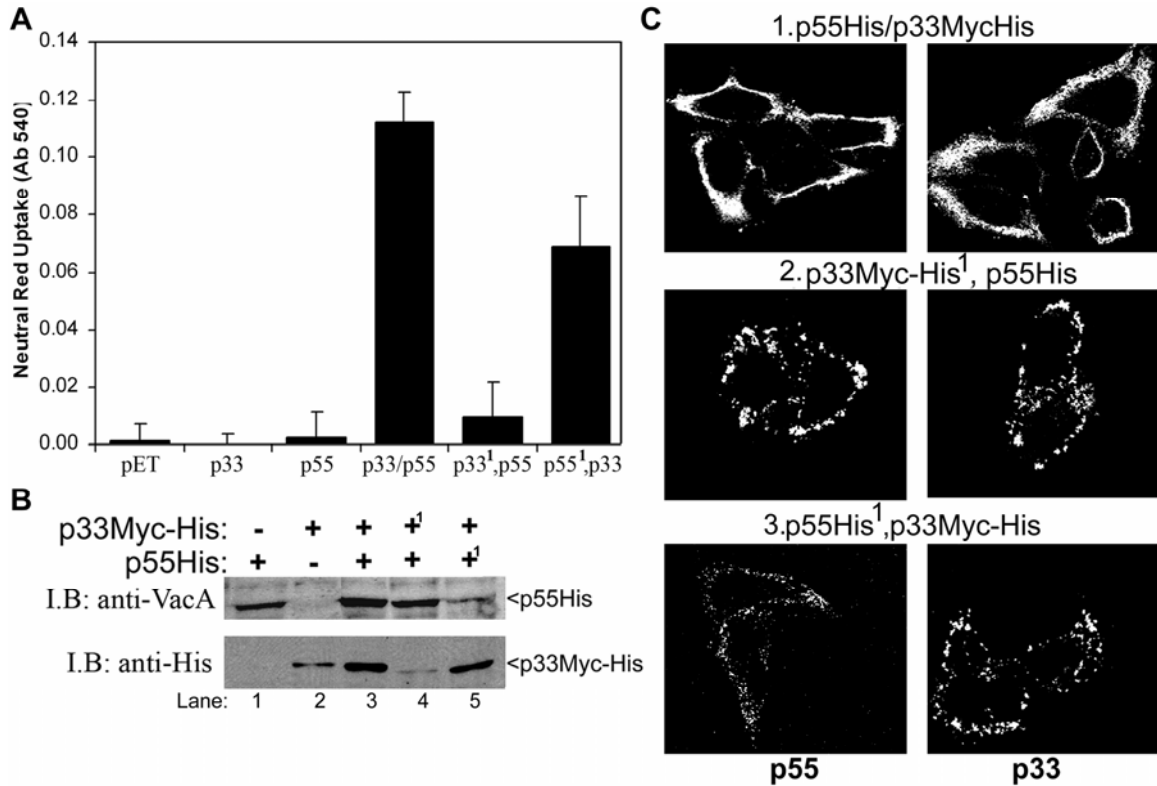
Figure 18. Sequential addition of p33 and p55 domains to HeLa cells. *A*) HeLa cells were intoxicated for 1 h at 37°C with the indicated *E. coli* soluble extracts. Alternatively, HeLa cells were sequentially intoxicated first with *E. coli* soluble extract containing p33 and then p55 (p33[1], p55), or p55 first and then p33 (p55[1], p33). In these sequential addition experiments the first sample ([1]) was incubated with cells for 1h at 37°C, then unbound proteins were washed away and the second sample was added for an additional 1h at 37°C. Input proteins were normalized by densitometry analysis of the immunoblot x-ray films, such that the relative concentrations of p33 and p55 domains were approximately equivalent. Vacuolating activity was measured by a neutral red uptake assay. Results represent the mean $\pm$ standard deviation from triplicate samples. These results indicate that the p33 and p55 VacA domains can exhibit vacuolating activity when added sequentially to mammalian cells (p55[1], p33). *B*) *E. coli* soluble extracts containing p55His and p33Myc-His were added to HeLa cells as indicated for 1h at 37°C. The capacity of the VacA proteins to interact with host cell membranes was assessed by immunoblot (I.B.) analysis using an anti-VacA polyclonal antibody to detect the p55 protein (top panel) and an anti-His antibody to detect the p33 protein (bottom panel). *C*) HeLa cells were intoxicated for 1h at 37°C with *E. coli* soluble extract containing a p33/p55 mixture (row 1). Alternatively, p33 was bound first followed by p55 (p33[1], p55; row 2), or p55 was bound first followed by p33 (p55[1], p33; row 3). The capacity of the VacA proteins to interact with the cell membrane of host cells was assessed by indirect immunofluorescence (I.F.) using an anti-VacA polyclonal antibody to detect p55His (p55; left panels) and an anti-c-Myc antibody to detect the p33Myc-His protein (p33; right panels).

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Internalization of sequentially added p33 and p55 VacA domains

We hypothesized that the lack of vacuolating activity when the p33 domain is added first followed by the p55 domain (p33[1], p55) is due to the lack of VacA internalization. To test this hypothesis, we analyzed the internalization of the p55 domain. As shown above (Fig. 17), no internalization of the p55 protein was detected when it was added alone to cells, but when the p55 and p33 proteins were mixed and added to cells, the p55 protein was internalized (p33/p55; Fig. 19). Similarly, when the p55 domain was added first to cells followed by the p33 domain (p55[1], p33), a condition that results in cell vacuolation (Fig. 18A), internalized p55 protein was detected. (p55[1], p33; Fig. 19). In contrast, when the p33 protein was added first followed by the p55 protein (p33[1], p55), a condition that fails to cause call vacuolation (Fig. 18A), the p55 protein was not detected inside cells (p33[1], p55; Fig. 19). Similar results were obtained when p33 was detected. These data indicate that in these sequential addition experiments, the order in which VacA domains are added to cells is an important determinant of whether VacA is internalized.
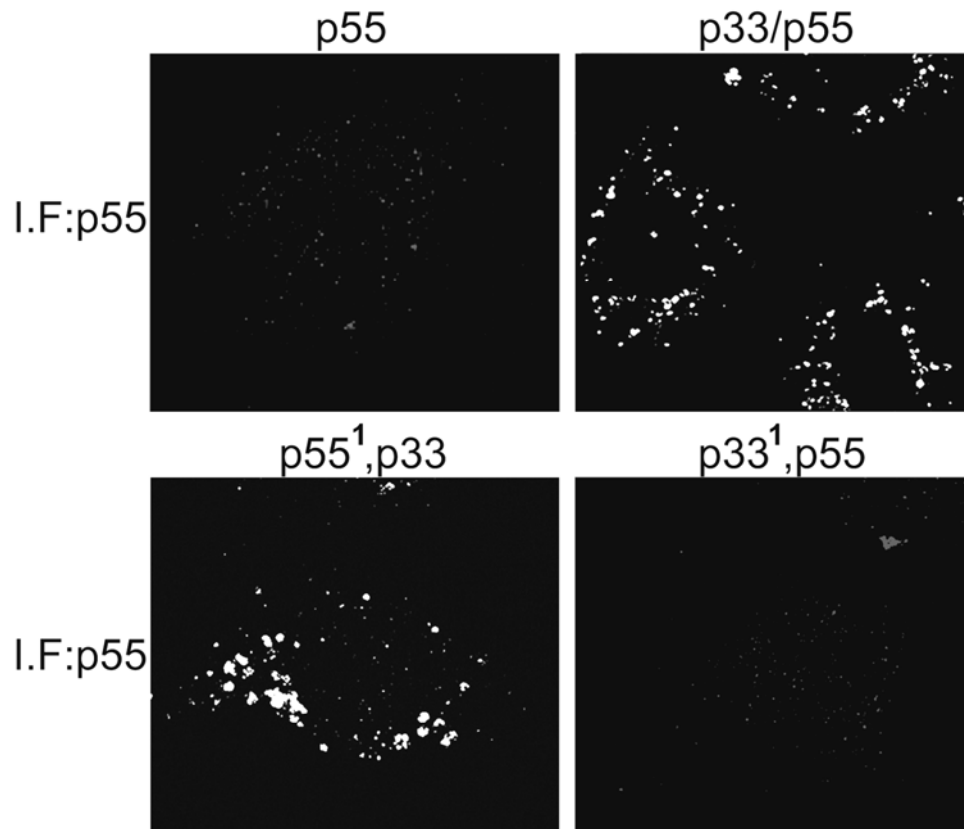
Figure 19.   Internalization of the p55 VacA domains. *A*) HeLa cells were intoxicated for 1h at 37°C with *E. coli* soluble extract containing the indicated samples. Then the media was replaced with fresh culture media and cells were incubated for an additional 16-24 h at 37°C. The ability of p55 to enter into cells was assessed by indirect I.F. of permeabilized cells using an anti-VacA polyclonal antibody to detect the p55 VacA domain. These data suggest that the p55 VacA domain is internalized into mammalian cells when both domains are present or when p55 is added first, followed by p33 (p55^1,p33). Sequential addition in the opposite order (p33^1,p55) did not lead to detectable VacA internalization.

Discussion

The *H. pylori* VacA toxin has the ability to induce an array of structural and functional alterations in intoxicated mammalian cells (58, 70). Previous studies have demonstrated that the mature secreted VacA toxin undergoes proteolytic degradation to yield two fragments (p33 and p55) (90, 94), but the relevant features of these two putative domains remain poorly characterized. In the current study, we expressed recombinant p33 and p55 VacA proteins, and sought to characterize functional properties of these two putative domains.

Our data indicate that the p33 and p55 VacA domains interact in solution to form protein complexes (Fig. 15A). This finding is consistent with the results of previous studies, which demonstrated that p33 and p55 proteins interact in the yeast two-hybrid system (CHAPTER II) (94), as well as in HeLa cells transiently co-transfected with plasmids expressing p33 and p55 (111). In addition, previous studies have shown that proteolysis of *H. pylori* VacA oligomeric complexes yields p33 and p55 fragments, which remain physically associated following proteolysis (CHAPTER II) (94). Notably, in the current study, heterotypic interactions between p33 and p55 were detected, but homotypic interactions (either p33/p33 or p55/p55) were not detected (Fig. 14A). This finding is consistent with the results obtained in a yeast two-hybrid system (CHAPTER II) (94). Heterotypic interactions between p33 and p55 have also been detected within cells using fluorescence resonance energy transfer (FRET) microscopy (107) or immunprecipitation methods (111), but whether p33 and p55 form homotypic complexes was not tested in these studies. Although homotypic interactions between p33 and p55 domains have not been detected in either the yeast two-hybrid system or in the current

study, it remains possible that such interactions could occur in the context of a membrane environment or within certain forms of VacA oligomers.

VacA 88 kDa monomers produced by *H. pylori* can assemble into large water-soluble oligomeric structures comprised of 6 to 14 subunits (1, 16, 46). Our data indicate that when mixed together, p33 and p55 domains form complexes composed of at least three independent subunits (Fig. 15B). However, we were unable to demonstrate assembly of p33 and p55 domains into high molecular mass oligomeric complexes similar to those formed by *H. pylori* VacA 88 kDa monomers (p88) (16). Moreover, when p33 and p55 proteins were mixed individually in solution with full-length p88 VacA from *H. pylori*, we were not able to detect interactions of either the p33 or the p55 domain with full-length p88 (Fig. 15C). In contrast, when the p33/p55 mixture is combined with p88-VacA, p33/p55/p88-VacA mixed oligomeric structures are formed (Fig. 15C). Collectively, these results suggest that formation of large VacA oligomeric structures proceeds more efficiently via interactions among p88-VacA monomers, rather than via interactions among isolated p33 and p55 domains.

The current studies indicate that p33 and p55 VacA domains are each independently able to interact with the surface of HeLa cells (Fig. 16A). Several previous studies have concluded that amino acid sequences in the p55 domain contribute to VacA binding to cells (32, 76, 101, 103), but potential interactions of the p33 domain with host cells have been analyzed in less detail. VacA binding to cells has been shown to be inhibited by antiserum reactive toward the p55 fragment but is not inhibited by antiserum reactive toward the p33 fragment (32). Also, inactive recombinant VacA proteins truncated at their C-terminus (p55 domain) have been shown to be impaired in

their ability to bind to mammalian cells (102). Furthermore, a truncated form of VacA containing mainly the p55 domain was reported to bind to target cells in a manner similar to full-length VacA (76). Interestingly, it has been shown previously that both the p55 and p33 domains are each able to associate with artificial lipid membranes in the absence of protein receptors (57), suggesting that each of these domains could potentially interact with lipids on the surface of mammalian cells. Whether p33 and p55 domains can each bind to the same component or different components on the surface of mammalian cells is not currently known.

An important conclusion of the current study is that VacA interactions with the surface of cells are altered in several ways when both p33 and p55 domains are present, compared to when only a single domain is present (Fig. 16). First, the total amount of VacA bound to cells increases substantially if both p33 and p55 are present (compared to only a single VacA domain) (Fig. 16A), and this increased binding is observed for both the p33 and p55 domain. Second, when p55 is added to cells in the absence of p33, the binding of p55 is detectable in immunoblot assays but not immunofluorescence assays (Fig. 16B). In contrast, if both p55 and p33 domains are added to cells, the binding of p55 is detectable in immunofluorescence assays (Fig. 16B). This suggests that the conformation or localization of the p55 domain on the surface of cells may be altered in the presence of the p33 domain. Finally, when added individually to cells, the p33 domain localizes in a punctate distribution on the cell surface, but when added to cells along with p55, p33 localizes in a continuous (non-punctate) distribution on the cell surface (Fig. 16B). The VacA interactions with the surface of cells that occur when both p33 and p55 domains are present are presumably relevant to subsequent localization of

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VacA within the cell. Specifically, we demonstrate that when added together, p33 and p55 are both internalized by host cells, whereas internalization is not detectable when p33 or p55 domains are added independently to host cells. The failure of p55 to be internalized when added independently to cells is consistent with a previous study in which it was shown that a p55 protein produced by *H. pylori* binds to the surface of host cells but is not internalized (76).

Another important conclusion of the current study is that p33 and p55 VacA domains lack detectable vacuolating activity when added individually to cells, but when mixed, the p33 and p55 domains complement each other for vacuolating activity (Fig. 13). Previous studies have shown that the p33 and p55 domains each lack detectable vacuolating cytotoxic activity when expressed intracellularly in transiently transfected cells, but that intracellular co-expression of these two domains results in vacuolating cytotoxic activity (20, 111, 113). The minimum portion of VacA capable of inducing vacuolating cytotoxic activity when expressed intracellularly comprises amino acids 1-422 (p33 together with amino acids 313-422 derived from p55) (113). Our current study indicates that the minimum portion of VacA capable of inducing vacuolating cytotoxic activity when added to the surface of cells is larger than that which is required when expressed intracellularly in transiently transfected cells (Fig. 14B). Specifically, we show that a VacA protein containing amino acids 1-700 possesses low levels of vacuolating cytotoxic activity when added to the surface of cells, and that further C-terminal truncations result in forms of VacA that have minimal or no detectable cytotoxic activity (Fig. 14C). The requirement of a larger portion of p55 for vacuolating activity when VacA is added to the surface of cells compared to when VacA is expressed intracellularly

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suggests that certain VacA amino acid sequences in this region (amino acids 422 to 821) may play an important role in the processes of VacA binding, internalization, or intracellular trafficking.

Further insight into the role of p33 and p55 domains comes from studies in which these domains are added sequentially to cells (Fig. 18 and 19). Binding of p55 to the cell surface followed by addition of p33 results in cell vacuolation, whereas addition of these domains in the opposite order fails to induce detectable cell vacuolation (Fig. 18A). There are numerous potential explanations for this result, but we favor a model in which p55 initially binds to a cell surface receptor, and subsequent addition of p33 leads to the formation of p55/p33 complexes, which are internalized by cells and produce vacuolating cytotoxic effects. The experimental results presented here provide evidence suggesting that binding of VacA to the surface of cells via the p55 domain can lead to internalization of the toxin and vacuolating cytotoxic effects, whereas binding via the p33 domain is not an effective mechanism for toxin internalization and does not produce detectable vacuolating cytotoxic effects (Fig. 18 and 19). We speculate that the p33 domain may bind to cell surface components that are different from those bound by p55, and that the cell surface component bound by p33 might not support internalization of the p33/p55 complexes (Fig. 19). Alternatively, this order of addition (p33[1],p55) may not permit the formation of the p33/p55 complexes in the proper conformation required for internalization.

VacA causes numerous effects on intoxicated cells, and many effects of VacA are dependent on the capacity of VacA to form membrane channels (17, 100, 106). Therefore, it is of interest to view the current results in the context of what is known

about functional domains of other pore-forming toxins. To our knowledge, VacA is the only pore-forming toxin that can be divided into two functional domains that complement each other for toxic activity when expressed individually. Two previous studies have investigated putative functional domains of the pore-forming toxins aerolysin (23) and listeriolysin (LLO) (24). In contrast to what is reported here with VacA, expression of separate domains of aerolysin and LLO, resulted in the production of proteins that lacked ability to complement each other for cytotoxic activity (23, 24). Interestingly, when these domains were co-expressed inside the bacteria, in both cases, the two domains were able to assemble into cytotoxic proteins, suggesting that assembly of functional toxins might require interactions between the two domains during early stages of the protein folding process (23, 24). The demonstration that VacA functional domains can complement each other for cytotoxic activity in solution suggests that there may be differences in the structural organization of VacA compared to most other known pore-forming toxins. Most pore-forming bacterial toxins act primarily on the plasma membrane of host cells, whereas VacA produces several effects that are dependent on its localization in intracellular sites. It seems likely that the unique feature of VacA may be related to the capacity of this toxin to exert multiple activities in multiple cellular sites.
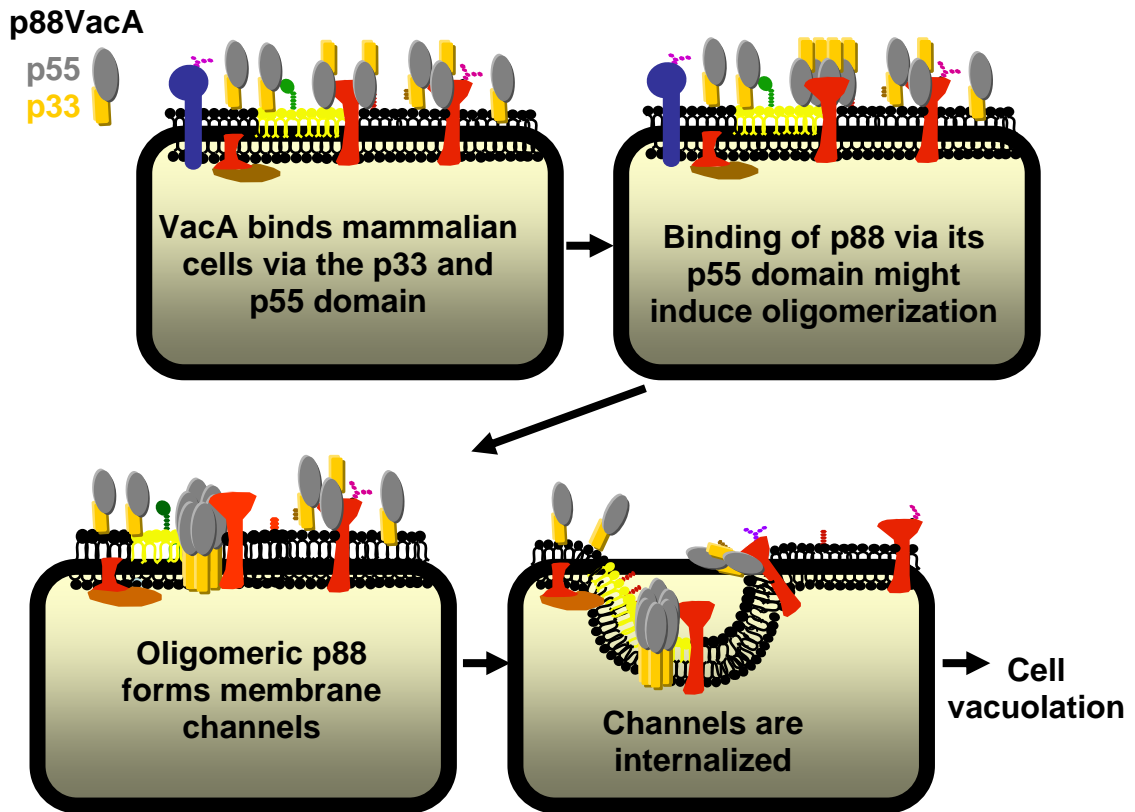
Figure 20. Model of VacA interaction with mammalian cells. Based on the data presented in this study, we propose a model in which VacA binds to mammalian cells via its p33 and p55 domains. Even though both domains can bind to cell surfaces, our data suggest that VacA binding via the p55 domain is important for VacA internalization and cytotoxic activity. We hypothesize that the VacA p55 domain binds to a receptor that is involved in the internalization of the surface-bound toxin. Previously it has been shown that lipid rafts play a role in the internalization of VacA. We hypothesize that binding of VacA via the p55 domain to lipid rafts or proteins within lipid rafts is required for the internalization of VacA channels, which in turns results in cell vacuolation.

CHAPTER IV

INHIBITION OF PRIMARY HUMAN T CELL PROLIFERATION BY
HELICOBACTER PYLORI   VACA TOXIN

Introduction

H. pylori can persistently colonize the human gastric mucosa for decades despite

the development of gastric mucosal inflammation, infiltration of immune cells to the

infection site, and specific antibody production. CD4$^+$ T cells have been shown to play a

critical role in protection against *H. pylori* infection (2, 22, 41, 45). Thus, it seems

possible that *H. pylori* may have evolved mechanisms to inhibit or modulate T cell

immunity. Indeed, recently it has been demonstrated that CagA (i.e. a protein that is

translocated into mammalian cells by the *H. pylori* type IV secretion system) and VacA

are able to modulate the immune system (12, 34, 56, 95). CagA has been shown to

inhibit the proliferation of interleukin-dependent B cells by suppressing JAK-STAT

signaling (95). VacA has also been shown to modulate the immune response by

inhibiting the activation of Jurkat T cells (a human T cell lymphoma/leukemia cell line)

as well as human peripheral blood lymphocytes (PBL), and to inhibit class II antigen

presentation (12, 34, 56). Studies in Jurkat T cells indicate that VacA blocks activation

of the nuclear factor of activated T cells (NFAT), a key transcription factor required for

optimal T cell activation (34). The process by which VacA inhibits NFAT activation in

Jurkat T cells is reportedly similar to the actions of the immunosuppressive drugs

cyclosporine A and FK-506, which inactivate the NFAT phosphatase calcineurin (34).

However, whether VacA inhibits the activation of primary human CD4$^+$ T cells has not

yet been studied in any detail. This chapter describes studies in which VacA effects on

primary human CD4$^+$ T cells were investigated.

Methods

Purification of VacA: *H. pylori* strains (wild-type strain 60190 and isogenic mutant strains) were grown as described before (16, 52, 100). Oligomeric forms of VacA were purified from broth culture supernatants of *H. pylori* as described previously (16). All experiments were performed using acid-activated preparations of VacA or acidified buffer control (PBS) (21), unless stated otherwise. The final VacA concentration was 10 μg/ml for all experiments, unless stated otherwise. For the dominant-negative assays (100), wild-type VacA was mixed with varying concentrations of VacA mutant toxins and the mixtures were acid-activated prior to addition of these samples to cells.

Primary human T cell purification and CFSE labeling: Resting CD4$^+$ human T cells were purified from healthy adult donors using magnetic bead purification (64, 96). The purified cells were 99% CD3$^+$CD4$^+$ as assessed by staining and flow cytometric analysis. Cell proliferation was monitored by labeling T cells with carboxy fluorescein diacetate succinimide ester (CFSE; Molecular Probes) prior to stimulation with α-CD3/α-CD28 antibodies. Purified cells were washed and resuspended in PBS. CFSE was added at a final concentration of 5 μM and incubated at 37°C for 3 min. Labeling was quenched by addition of 50% fetal calf serum in PBS, followed by three washes with RPMI-supplemented media. All CFSE labeling and cultures were performed in the dark.

Activation of primary human T cells: Purified resting T cells were activated by cross-linking with plate-bound anti-CD3 antibody (OKT-3, ATCC) and soluble anti-

CD28 antibody (BD Biosciences). The plates were first coated with anti-mouse IgG (10 μg/ml, Caltag), followed by anti-CD3 antibody. Cells were removed from the activation signals after 48 hours and expanded in media supplemented with recombinant human IL-2 (Chiron, 200 U/ml). Jurkat T cells were TCR/CD28-stimulated as described above, or with phorbol myristate acetate (PMA, 50 ng/ml; Sigma) and ionomycin (500 ng/ml; Sigma), and maintained in RPMI media containing 10% FCS. To inhibit TCR/CD28 stimulation or IL-2-driven stimulation, T cells were treated with cyclosporine A (50 nM, Alexis Biochemicals), FK506 (100 nM, Alexis Biochemicals), or rapamycin (200 ng/ml, Alexis Biochemicals) respectively.

FACS Analysis and IL-2 detection: IL-2 receptor (CD25) surface expression was detected by staining with phycoerythrin (PE)-conjugated anti-human CD25 (BD Biosciences). Stained and CFSE-labeled cells were fixed with 1% paraformaldehyde and the data was acquired on a FACSCalibur flow cytometer and analyzed using the Cellquest software (BD Biosciences). Forward- and side-scatter profiles were used to define the live population. IL-2 secretion into culture supernatants was determined using cytometric bead array (CBA) according to the manufacturer's instructions (BD Biosciences), and analyzed using CBA 6-bead analysis software (BD Biosciences). Samples were analyzed on a FACSCalibur® four color cytometer, using the CellQuest program.

Generation and use of NFAT reporter primary human T cells: To generate a primary human CD4^{+} T cell line that expressed an NFAT transcriptional reporter, three

tandem copies of the NFAT binding site of the IL-2 promoter (29) were sub-cloned upstream of the EGFP gene (Clontech) to direct its transcription (64). This NFAT-GFP expression cassette was then cloned into a lentiviral vector in reverse orientation and VSV-G pseudotyped viruses were generated via co-transfection of 293 T cells (64). Activated primary CD4$^+$ T cells were transduced with these pseudotyped viruses at a suboptimal MOI. After 7 days, cells constitutively expressing GFP were removed by FACS sorting and GFP-negative cells were TCR/CD28- or PMA/Ionomycin-stimulated for 24 hours. NFAT-GFP T cells that up-regulated GFP upon activation were positively sorted by flow cytometry and further expanded in IL-2-supplemented media. Following enrichment, at least 10% of the NFAT-GFP T cell population displayed GFP-inducible expression upon TCR stimulation.

Cell cycle analysis of T cell proliferation: Day 4 TCR/CD28-stimulated T cells were washed to remove exogenous IL-2 and maintained in IL-2 free medium for 24 hours to synchronize cells at phase G_1 of the cell cycle. During this period, the cells were treated with VacA or other additives. Following incubation in IL-2-containing medium for the indicated times, cells were subjected to propidium iodide (PI) staining and analyzed by flow cytometry (73). To evaluate the kinetics of DNA replication, T cells were washed to remove IL-2, and treated with VacA for 8 hours. Following VacA treatment, cells were incubated for 24 hours in IL-2-containing medium. Then 10μM 5-bromo-2-deoxyuridine (BrdU) was added to the cultures, and cells were harvested at different time points for BrdU antibody staining with a commercially available kit (BD Biosciences), according to the manufacturer's instructions.

VacA inhibits activation-induced proliferation of primary human CD4$^{\pm}$ T cells

Previous reports have indicated that VacA inhibits activation of T cells, particularly a transformed T cell line (Jurkat T cells) (12, 34). While several experiments were performed on peripheral blood lymphocytes (PBL) (34), the mode of VacA action on purified primary T cells was not assessed. To investigate whether VacA inhibits the activation of primary T cells in a manner similar to its effect on transformed cells, we purified CD4^{+} primary human T helper (T_h) cells from peripheral blood mononuclear cells (PBMC) of healthy individuals and labeled them with CFSE, a cell-permeable dye that allows for the quantification of cell division within a population. CFSE-labeled T_h cells were then pre-treated with medium, PBS, or VacA, followed by TCR and CD28 stimulation using α-CD3 and α-CD28 antibodies (TCR/CD28 stimulation) as described in the Methods section. As expected, T_h cells pre-treated with medium or PBS rapidly proliferated, resulting in up to 7 divisions when analyzed at 5 days-post TCR/CD28 stimulation (Fig. 21A and B). In contrast, treatment with VacA potently inhibited the proliferation of T_h cells (Fig. 21A and B) in a dose-dependent manner (Fig. 21C). Incubation of purified VacA at acid pH (pH $\leq$4.5; a process termed acid-activation) markedly enhances the capacity of the toxin to undergo internalization and cause vacuolating cytotoxic effects in mammalian cells (21, 53). Accordingly, acid-activation markedly enhanced the capacity of VacA to inhibit T_h cell proliferation (Fig. 21D).
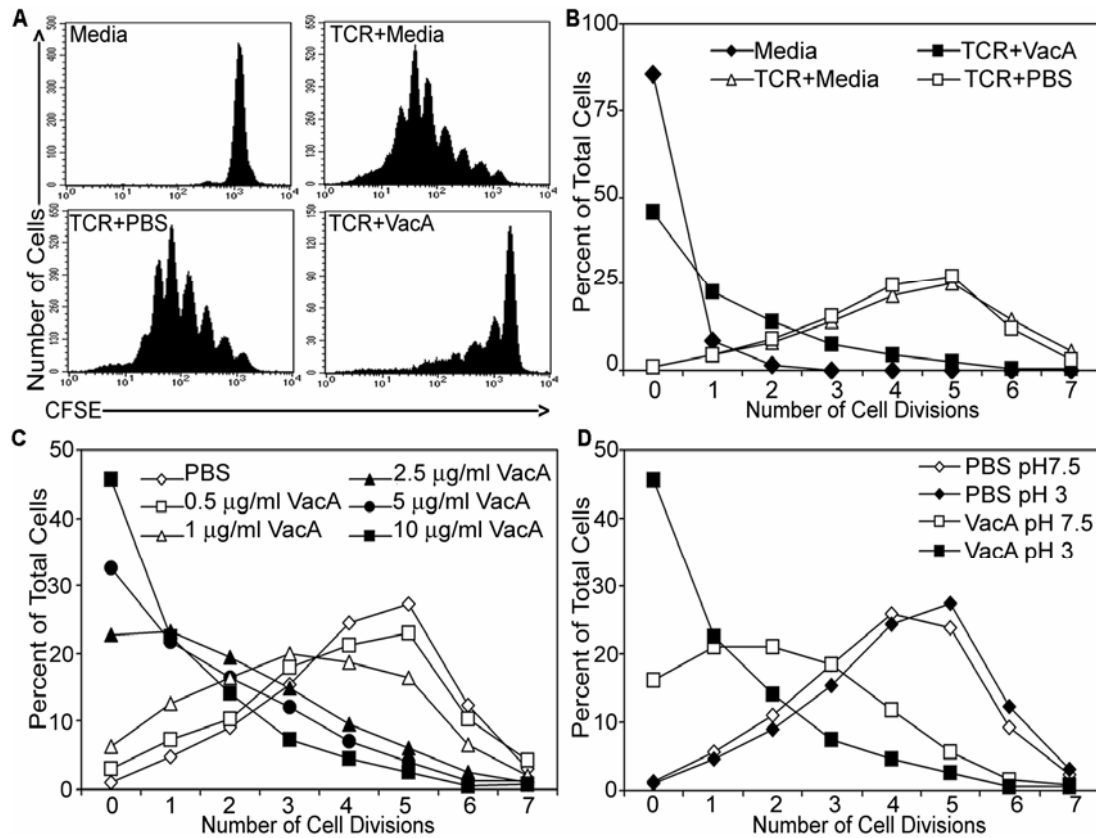
Figure 21. VacA inhibits activation-induced proliferation of primary human CD4$^+$ T$_h$ cells. *A*) purified primary human T$_h$ cells were labeled with CFSE and treated with acid-activated VacA (10 μg/ml), acidified-PBS (PBS), or medium alone for 1 hour, followed by TCR/CD28 stimulation for 48 hours as described in the Methods section. Control cells were treated with medium alone, without TCR/CD28 stimulation. Activated T cells were expanded in IL-2-containing media and T cell proliferation was analyzed at day 5 post-activation by flow cytometry. *B*) graphic representation of the histograms shown in panel *A*. *C*) dose-response analysis of VacA effects on primary human CD4$^+$ T cell proliferation. T$_h$ cells were CFSE-labeled and treated with different concentrations of acid-activated (pH 3) VacA for 1 hour. Cells were then stimulated and analyzed as in panel *A*. *D*) effects of acid-activated VacA (pH 3) and non-acid-activated VacA (pH 7.5) on T cell proliferation. T$_h$ cells were CFSE-labeled and treated with acid-activated or non-activated VacA (10 μg/ml) as described above. All the results are representative of three experiments using cells from different donors and different toxin preparations.

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The N-terminal hydrophobic domain of VacA is required for inhibition of primary human T_h cell proliferation

Most of VacA effects on mammalian cells have been shown to be dependent of the ability of VacA to form membrane-channels (17, 100, 106). Structure-function analyses have revealed that an intact structure of a hydrophobic domain within the VacA amino-terminal region is required for the formation of anion-selective membrane channels (Table 1) (52, 100). We hypothesized that the formation of VacA anion-selective channels may play an important role in the process by which VacA inhibits proliferation of activated T cells. To further test this hypothesis, we examined the effects of three VacA mutant toxins, two of them contain single amino acid substitutions in the hydrophobic domain and one contains an in-frame deletion of the hydrophobic domain of VacA (VacA-P9A, VacA-G14A, and VacA-$\Delta\{6-27\}$ respectively) on proliferation of primary human T_h cells (Table 1). These VacA mutant proteins are defective in channel-forming activity, but retain other structural and functional characteristics of the wild-type VacA protein, including the ability to form oligomeric structures and the capacity to bind and enter cells (Table 1) (52, 100, 106). In contrast to wild-type VacA, these mutant proteins did not cause any detectable inhibition of T cell proliferation (Fig. 22). These data indicate that an intact VacA amino-terminal hydrophobic domain is required for VacA-mediated inhibition of T cell proliferation, and suggest that the formation of VacA anion-selective membrane channels is important for the suppression of activation-induced proliferation of primary T_h cells.

Table 1. Characterization of VacA mutant toxins used in this study.

VacA Toxins	Oligomer formation [a]	Binding / internalization [b]	Channel formation [c]	Cytotoxic Activity [d]
WT [e]	+	+	+	+
Δ6-27	+	+	-	-
P9A	+	+	-	-
G14A	+	+	-	-

[a] Oligomer formation of VacA toxins was analyzed by determining whether these proteins eluted as large oligomeric structures (~1,000 kDa) from a gel-filtration chromatography column (16, 52, 100).

[b] Binding and internalization of VacA toxins was assessed by indirect immunofluorescence analysis using HeLa cells (106).

[c] VacA channel activity was assessed using planar lipid bilayers (52, 100).

[d] Toxins (20 µg/ml) that induced vacuolation in more than 50% of HeLa cells were scored positive for cytotoxic activity (52, 100).

[e] WT, wild-type VacA from *H. pylori* strain 60190.
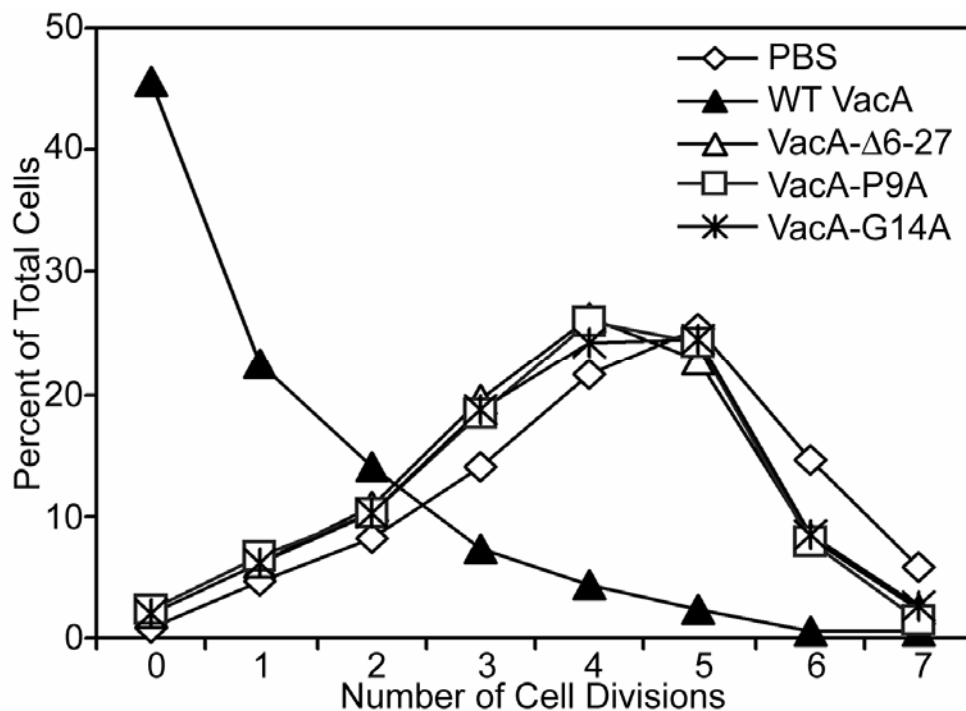
Figure 22. Analysis of VacA mutant proteins demonstrates that an intact N-terminal hydrophobic domain is required for VacA-mediated effects on T cell proliferation. Purified primary human T_h cells were CFSE-labeled and treated with wild-type VacA (WT VacA, 10 μg/ml), one of 3 different mutant toxins (each 10 μg/ml), or PBS for 1 hour. Cells were then TCR/CD28 stimulated for 48 hours, expanded in IL-2-containing media, and analyzed by flow cytometry at day 5 after stimulation as described in the Methods section. Results are representative of three experiments using cells from different donors and different toxin preparations.

Effects of VacA on IL-2 secretion in Jurkat T cells and primary human T cells

It has been reported recently that VacA blocks the secretion of interleukin-2 (IL-2) by mitogen-stimulated Jurkat T cells (34). Therefore, we investigated whether VacA also inhibits IL-2 secretion in primary T_h cells. In agreement with a previous report (34), we found that wild-type VacA potently suppressed IL-2 secretion in Jurkat T cells, very similar to the immunosuppressive drugs cyclosporine A and FK506, regardless whether the cells were TCR/CD28-stimulated or stimulated with PMA and ionomycin (Fig. 23A). Notably, a VacA mutant toxin deficient in vacuolating cytotoxic activity and defective in its ability to inhibit primary T_h cells proliferation (Fig. 22; VacA Δ6-27) did not inhibit IL-2 secretion in Jurkat T cells (Fig. 23A). In contrast to the marked inhibitory effect of wild-type VacA on IL-2 secretion by Jurkat T cells, VacA had only a modest effect on IL-2 secretion by primary human T_h cells (Fig. 23A). To investigate if VacA might selectively inhibit IL-2 secretion in naïve or memory T cell subsets, we compared levels of IL-2 secreted by FACS-sorted naïve (CD45RA$^+$/CD45RO$^-$) and memory (CD45RA$^-$/CD45RO$^+$) CD4$^+$ T cells following treatment with VacA and TCR/CD28-stimulation. VacA treatment resulted in no detectable reduction of IL-2 secretion by either naïve or memory T cell subsets, whereas cyclosporine A treatment effectively inhibited IL-2 secretion by both CD4$^+$ subsets (Fig. 23B). TCR/CD28 stimulation of primary human T cells induces the expression of both IL-2 and the high affinity IL-2 receptor α-chain (CD25). Therefore, we next investigated whether VacA treatment inhibited the surface-expression of CD25. Primary human T_h cells were pre-treated as described above and CD25 expression was analyzed at 24 h post-TCR/CD28 stimulation by flow cytometric analysis. No significant difference was detected in the CD25 expression of VacA-treated

primary human T cells and cells treated with PBS or medium (Fig. 23C). These data suggest that VacA-mediated inhibition of activation-induced proliferation of primary human CD4$^+$ T cells is not due to effects on IL-2 secretion or cell surface expression of CD25.
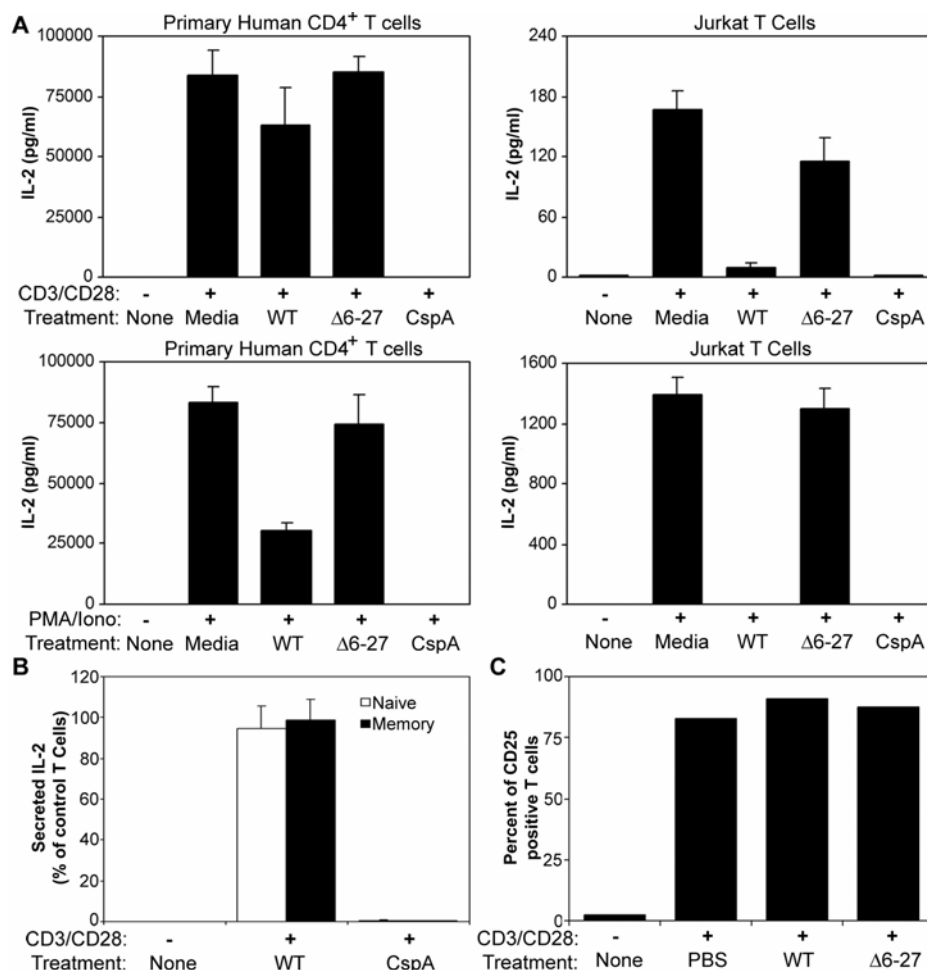
Figure 23. VacA-treated Jurkat T cells secrete diminished levels of IL-2, whereas VacA-treated primary human T cells continue to secrete high levels of IL-2.
A) purified primary human T_h cells (right columns) or Jurkat T cells (left columns) were pre-treated with medium alone, wild-type (WT) VacA, VacA-Δ(6-27), or cyclosporine A (CspA) for 1 hour, followed by TCR/CD28 stimulation (CD3/CD28; top panels) or stimulation with PMA (50 ng/ml) and ionomycin (500 ng/ml) (bottom panels), as indicated. IL-2 secretion was measured at 24 h after stimulation using a cytometric bead array (CBA) as described in the Methods section. Results represent the mean $\pm$ S.D. from triplicate samples. *B*) Purified primary human T_h cells were FACS-sorted into naïve (CD45RA$^+$/CD45RO$^-$) and memory (CD45RA$^-$/CD45RO$^+$) CD4$^+$ T cell subsets. Purified cells were pre-treated as indicated for 1 hour, followed by TCR/CD28 stimulation (CD3/CD28). IL-2 secretion was as described in panel A. Results represent the mean $\pm$ S.D. from duplicate samples from two different donors, and are expressed as the percentage of IL-2 secreted by the control cells pre-treated with medium. *C*) Purified primary human T_h cells were pre-treated as indicated, followed by TCR/CD28 stimulation (CD3/CD28). IL-2 receptor α-chain (CD25) surface expression was detected 24 h after stimulation by staining with phycoerythrin (PE)-conjugated anti-human CD25 (BD Biosciences). Results are expressed as the percentage of surface expressed CD25 in control cells pre-treated with medium.

VacA inhibits primary human T cell proliferation through an NFAT-independent mechanism

To further confirm that the lack of proliferation of VacA-treated primary T_h cells was not due to insufficient IL-2 levels in the medium, the cultures were supplemented with saturating concentrations of exogenous recombinant IL-2 (200 U/ml) during TCR/CD28 stimulation. VacA potently inhibited the proliferation of primary T_h cells, even in the presence of excess exogenous IL-2 (Fig. 24A). Thus, together with data presented in figure 20, we conclude that the VacA-mediated inhibition of primary human T_h cell proliferation cannot be attributed to VacA effects on IL-2 secretion.

VacA has been shown to inhibit NFAT activation in Jurkat T cells, resulting in a loss of IL-2 secretion (34). However, since VacA has only a modest effect on IL-2 secretion and CD25 surface-expression in primary T cells (Fig 23B and C), we hypothesized that VacA-mediated inhibition of primary human T cell proliferation may occur via an NFAT-independent mechanism. To test this hypothesis, we developed a primary human T_h cell stably transduced with a lentiviral vector in which the NFAT binding site of the IL-2 promoter drives the expression of the green fluorescent protein (NFAT-GFP T cells) (64). NFAT-GFP T cells were treated with wild-type VacA, PBS, cyclosporine A, or VacA-Δ(6-27) for 1 hour prior to TCR/CD28 stimulation. GFP expression was then analyzed 24 hours-post stimulation via flow cytometric analysis. As expected, GFP expression was induced upon TCR/CD28 stimulation of NFAT-GFP T cells (Fig. 24B). In the presence of cyclosporine A, which is potent inhibitor of NFAT activation, stimulated NFAT-GFP T cells did not express GFP (Fig. 24B). Stimulated cells treated with VacA expressed GFP similar to the PBS-treated cells, regardless whether the primary cells were TCR/CD28-stimulated or stimulated with

PMA/ionomycin (Fig. 24B). These results, taken together with our findings that VacA causes only modest effects on IL-2 secretion in primary T_h cells, suggest that VacA can inhibit proliferation of primary human T_h cells via an NFAT-independent mechanism.
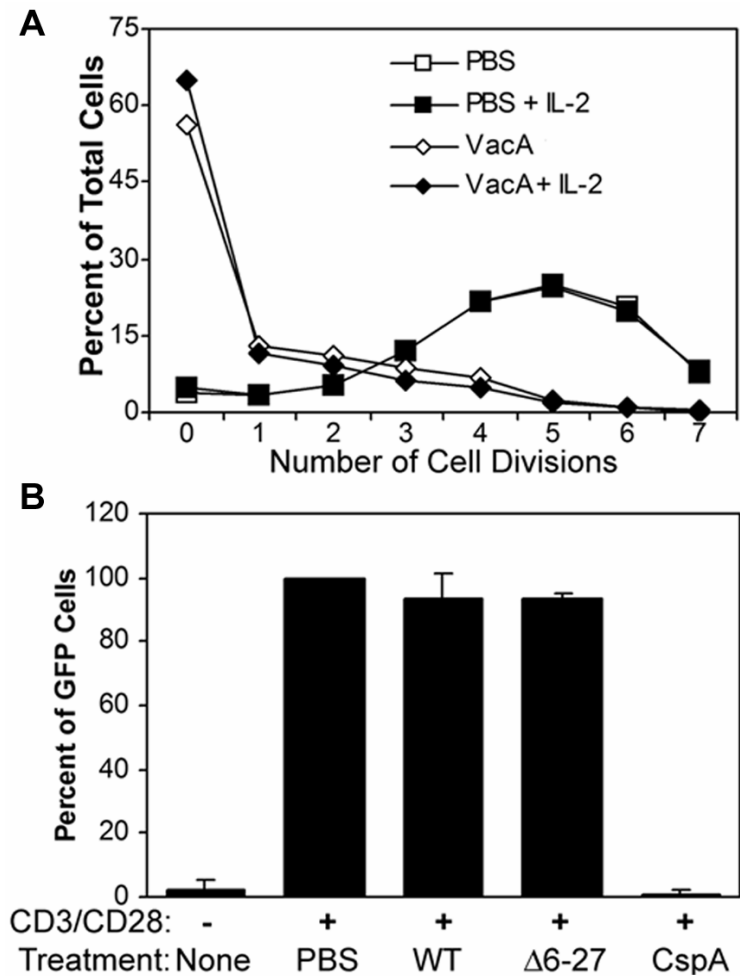
Figure 24. VacA inhibits activation-induced proliferation of primary human T_h cells independent of effects on IL-2 secretion and NFAT activation. *A*) purified primary human T_h cells were CFSE-labeled and pre-treated with wild-type VacA or PBS in the presence or absence of supplemental IL-2 (200 U/ml) for 1 hour as indicated. Cells were then TCR/CD28 stimulated in the presence or absence of supplemental IL-2 for 48 h, expanded in IL-2 supplemented media, and subjected to flow cytometric analysis at day 5 post-treatment. *B*) primary human $CD4^+$ T cells stably transduced with a GFP reporter under the control of NFAT (NFAT-GFP T_h cells; see Methods section) were pre-treated with the different additives as in panel *A* for 1 hour prior to TCR/CD28 stimulation (CD3/CD28). GFP expression was assessed by flow cytometric analysis 24 hours after stimulation. Results represent the mean $\pm$ S.D. from triplicate samples and are expressed as the percentage of cells demonstrating inducible expression of GFP, relative to the PBS-treated cells. CspA, cyclosporine A (50 nM); WT, wild-type VacA toxin (10 µg/ml); and Δ6-27, VacA-Δ(6-27) mutant toxin (10 µg/ml).

VacA inhibits IL-2-driven proliferation of primary human T_h cells, but not IL-2-dependent survival

To gain further insight into the mechanism employed by VacA to suppress activation-induced proliferation of primary T cells, we investigated the kinetics of VacA-mediated effects. CFSE-labeled primary human T_h cells were TCR/CD28-stimulated as previously described, and treated with PBS, wild-type VacA, or VacA-Δ(6-27) at different time points after stimulation. T cell proliferation was assessed 5 days after stimulation by flow cytometric analysis. VacA inhibited proliferation of T_h cells even when added 48 hours after stimulation, suggesting that VacA effects are largely independent of early TCR/CD28 signals (Fig. 25A).

We next tested whether VacA blocked IL-2-dependent proliferation of primary T cells at later time points (96 hours) after stimulation, a stage in which T cell proliferation and survival are solely dependent on IL-2 signals, but not on TCR/CD28 signals (27). For these experiments, T_h cells were TCR/CD28-stimulated for 48 hours and expanded in the presence of IL-2 for an additional 2 days. At day 4 post-activation, T cells were removed from IL-2, and treated with VacA or other additives for 24 hours. IL-2 was then added back to the medium and cells were expanded for an additional 3 days. Cell counts were performed to assess cellular proliferation at day 3 post-IL-2 stimulation. As expected, activated T cells treated with PBS and stimulated with IL-2 proliferated approximately 6-fold from day 0 to day 3, and no proliferation was observed in the absence of IL-2 (Fig. 25B). In the presence of VacA, however, T cell numbers only increased about 3-fold (p < 0.001), similar to cells treated with rapamycin, an immunosuppressive drug that blocks IL-2 driven proliferation of T cells (Fig. 25B) (14,

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75). These data indicate that VacA inhibits IL-2-driven proliferation of activated primary human T_h cells.

IL-2 signals are required not only for activation-induced T cell proliferation, but also for survival of these cells (27). Therefore, we also monitored the viability of the activated T_h cells cultured in the presence or absence of VacA. As expected, the majority of activated T cells (>90%) incubated without IL-2 for 3 days underwent apoptosis (Fig. 25C). In contrast, VacA treatment did not result in a significant increase in cell death (Fig. 25C), suggesting that VacA inhibits IL-2-driven proliferation without altering IL-2-dependent survival.
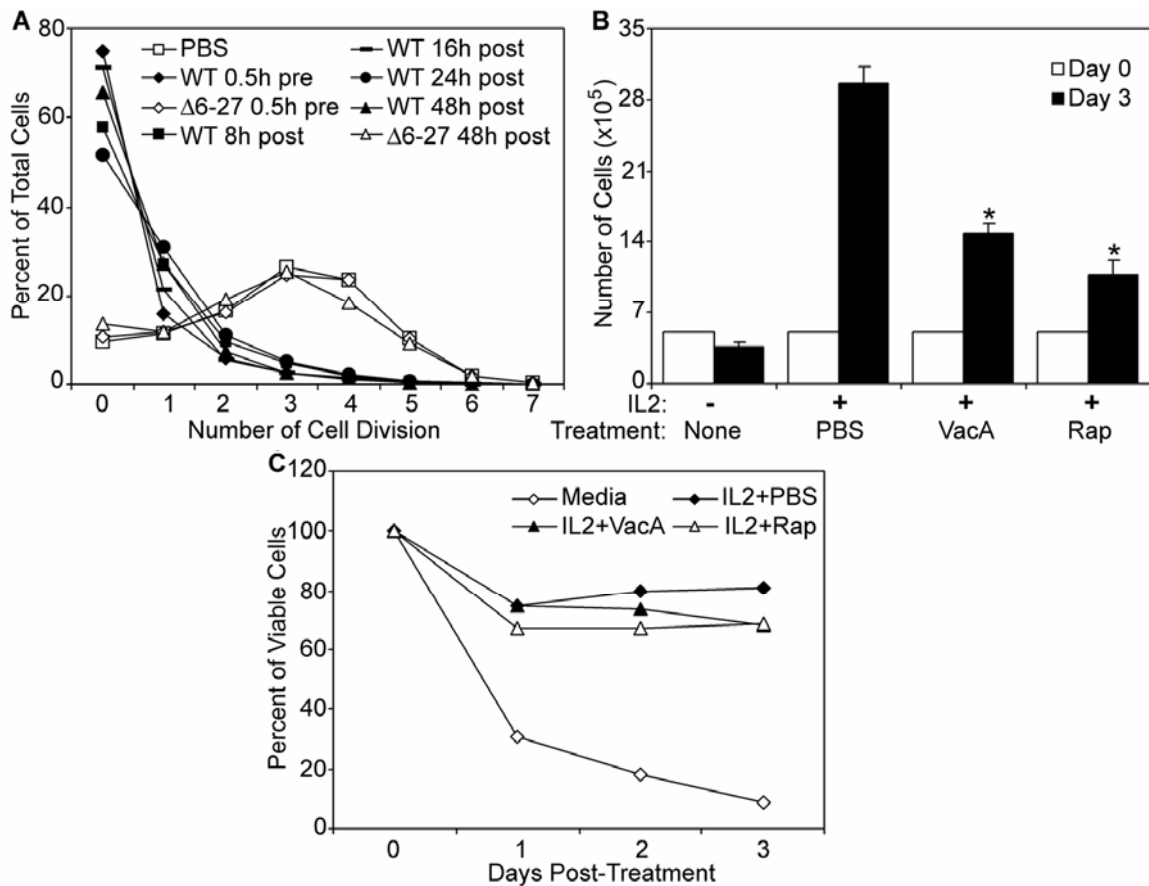
Figure 25. VacA inhibits IL-2-driven proliferation of primary human T$_h$ cells. *A*) wild-type (WT) VacA (10 µg/ml) or VacA-Δ(6-27) (10 µg/ml) were added to CFSE-labeled purified primary human CD4$^+$ T cells at the indicated time points either preceding or following (pre or post) TCR/CD28 stimulation. Activated cells were expanded in IL-2-containing media and cell proliferation was analyzed by flow cytometry at day 5 after stimulation. *B*) primary human T$_h$ cells were TCR/CD28 stimulated for 48 hours and expanded in the presence of IL-2 for 2 additional days. At day 4 after stimulation, T cells were removed from IL-2 and treated with PBS, wild-type VacA (10 µg/ml), or rapamycin (Rap; 200 ng/ml) for 24 hours. After 24 hours, IL-2 was added back to the media as indicated and cells were treated again with the different additives and expanded in fresh media containing supplemental IL-2 for 3 days. Cell proliferation was assessed by cell counting with a hemacytometer. Results represent the mean $\pm$ S.D. from triplicate samples. * = p < 0.001 when compared to the PBS-treated cells. *C*) viability of T$_h$ cells was determined by flow cytometric gating for viable cells based on forward and side scatter properties at day 1, 2, and 3 after TCR/CD28 stimulation.

VacA attenuates IL-2-dependent cell cycle progression in primary human T_h cells

To determine whether VacA-mediated inhibition of IL-2-driven proliferation is due to a perturbation of cell cycle progression, we assessed cell cycle progression of activated T cells in the presence of VacA. Primary human T_h cells were TCR/CD28-stimulated and expanded in IL-2-containing medium as described above. Day 4-activated T_h cells were removed from IL-2 for 24 hours to induce a G_1-phase cell-cycle arrest (62), and were then treated with VacA or other additives. IL-2 was then added back to the medium, and cell cycle distribution was analyzed at different time points through propidium iodide (PI) staining. The activated T_h cells arrested in G_1-phase re-entered the cell cycle approximately 24 to 36 hours after IL-2 stimulation, as seen by the increased number of T_h cells in S and G_2M phases (Fig. 26). In contrast, cells treated with either rapamycin (which inhibits IL-2-induced cell cycle progression by arresting the cells at the G_1-phase) (14, 62, 75)or VacA displayed impaired IL-2-induced cell cycle progression (Fig. 26). Taken together, these data provide strong evidence that VacA inhibits IL-2-driven proliferation in activated primary human T_h cells by suppressing cell cycle progression.
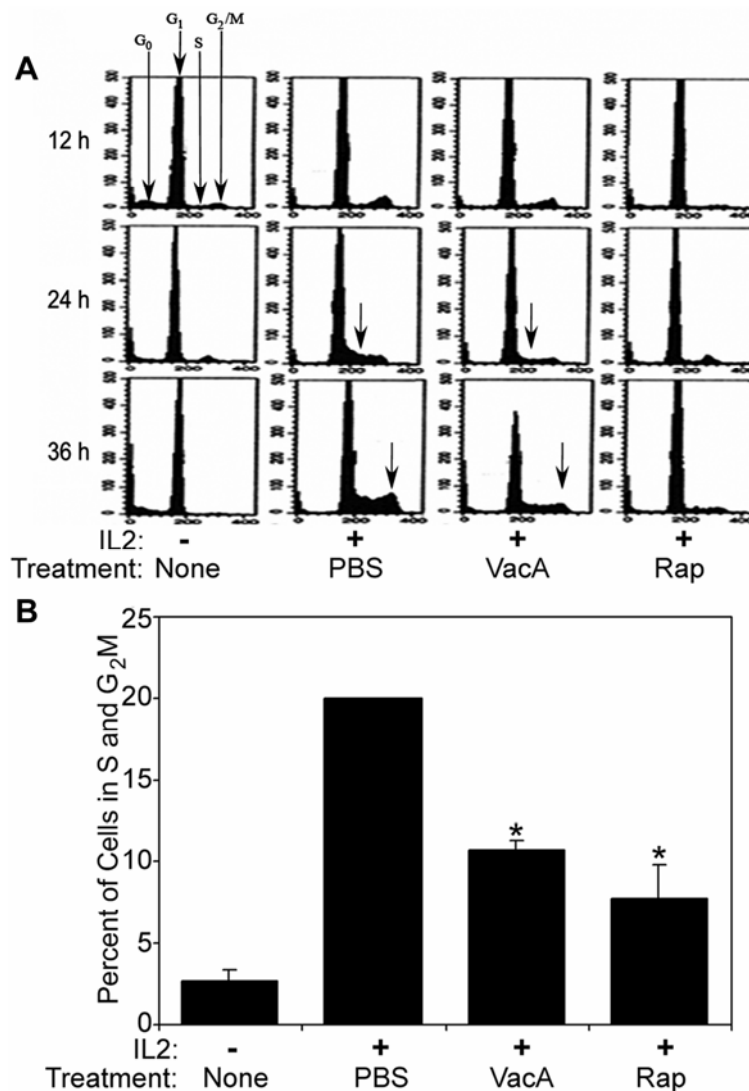
Figure 26. VacA delays IL-2-driven cell cycle progression. *A)* Propidium iodide (PI) staining Histograms of primary human T_h cells. Primary human T_h cells were TCR/CD28 stimulated for 48 hours and expanded in the presence of IL-2 for 2 additional days. At day 4 after stimulation, T cells were removed from IL-2 and treated with PBS, wild-type VacA (10 µg/ml), or rapamycin (Rap; 200 ng/ml) for 24 hours. After 24 hours, IL-2 was added back to the media as indicated and cells were treated again with the different additives and expanded in fresh media containing supplemental IL-2 for 3 days. Cell-cycle distribution was analyzed at 12, 24, and 36 h following IL-2 stimulation using and flow cytometry analysis. *B)* The percentages of total cells in S-phase and G_2M phase 36 h post IL-2 stimulation are shown. Results represent the mean $\pm$ S.D. from triplicate samples. * = $p < 0.01$ when compared to the PBS treated cells. Results are representative of at least two experiments using cells from different donors and different toxin preparations.

VacA-Δ(6-27) inhibits the immunosuppressive effects of wild-type VacA on T cells

In studies of VacA-induced effects on HeLa cells and AGS cells (a human gastric epithelial cell line), the VacA mutant toxin (VacA-Δ6-27) has been reported to exhibit a dominant-negative phenotype (100). When mixed in an equimolar ratio with wild-type VacA, VacA-Δ(6-27) blocks the capacity of wild-type VacA to cause cell vacuolation (100), form anion-selective membrane channels (100), induce cytochrome c release (106), and induce apoptosis (17). The inhibitory actions of VacA-Δ(6-27) are thought to be due to the formation of inactive mixed-oligomeric complexes, comprised of both wild-type and mutant toxin (100). To investigate whether VacA-Δ(6-27) could block the actions of wild-type VacA on T_h cells, the two toxins were mixed and added at various stoichiometric ratios to CFSE-labeled, resting T_h cells. CFSE-labeled primary human T_h cells were subsequently TCR/CD28 stimulated, and proliferation was evaluated by flow cytometric analysis 5 days post-stimulation. VacA-Δ(6-27) potently blocked wild-type VacA-mediated inhibition of T cell proliferation in a dominant-negative fashion, since it was partially effective even in the presence of 20-fold molar excess of wild-type VacA (Fig. 27A). VacA-Δ(6-27) also blocked the inhibitory effects of wild-type VacA on IL-2 secretion in Jurkat T cells (Fig. 27B), suggesting that the VacA-Δ(6-27) is able to block VacA effects on T cells.
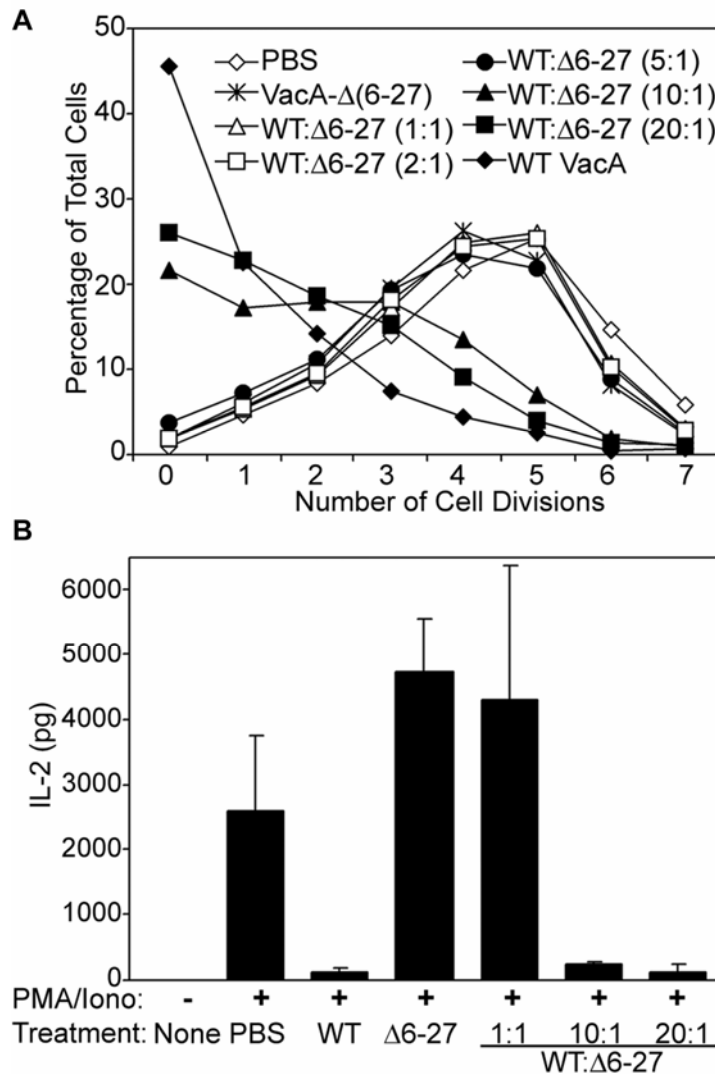
Figure 27. Effects of a dominant-negative mutant VacA toxin.
A) primary human T_h cells were CFSE-labeled and then treated for 1 h with wild-type VacA and VacA-Δ(6-27) in different ratios (WT:Δ6-27) as indicated. Cells were then TCR/CD28 stimulated and expanded in IL-2-supplemented media. Cell proliferation was analyzed by flow cytometric analysis on day 5 after stimulation. *B*) Jurkat T cells were pre-treated with wild-type VacA and VacA-Δ(6-27) as in panel *A* for 1 h as indicated, activated with PMA and ionomycin, and then incubated for 24 h. Culture supernatants were assayed for IL-2 secretion using a cytometric bead array (CBA) assay as described in the Methods section. Results are representative of three experiments using cells from different donors, different preparations of Jurkat T cell lines, and different toxin preparations.

Discussion

In this study we show that VacA inhibits the proliferation of TCR/CD28-stimulated primary human $CD4^+$ T helper (T_h) cells (Fig. 18). In addition, our data demonstrate a qualitative difference between VacA-mediated effects on IL-2 secretion by Jurkat T cells and primary human T_h cells (Fig. 20). In contrast to the potent inhibitory effects of VacA on IL-2 secretion in Jurkat T cells (Fig. 23A), VacA-treated primary T cells retain the capacity to secrete high levels of IL-2 (Fig. 23A). The suppressive effect of VacA on Jurkat T cell proliferation has previously been attributed to suppression of IL-2 expression, occurring at the level of transcriptional regulation due to NFAT inhibition (34). In contrast, our results provide evidence that VacA-mediated inhibition of primary human T_h cell proliferation occurs at a later stage of T cell activation, wherein the immunosuppressive drugs cyclosporine A and FK-506 (NFAT-activation inhibitors) are no longer inhibitory (Fig. 24). Specifically, we show for the first time that VacA impairs IL-2-driven cell-cycle progression in activated primary human T_h cells (Fig. 25), resulting in an inhibition of IL-2-driven proliferation. This effect of VacA resembles the actions of the immunosuppressive drugs rapamycin and sanglifehrin A (14, 114), two known inhibitors of IL-2-driven proliferation.

We also show, based on analysis of single-point mutant toxins (Table 1), that the inhibitory effects of VacA on primary human T_h cell proliferation and IL-2 secretion in Jurkat T cells are dependent on the integrity of the VacA N-terminal hydrophobic domain, which is required for formation of membrane channels (Fig. 22) (52, 100). This provides strong evidence that VacA-mediated effects on T cell proliferation are dependent on the formation of membrane channels. This conclusion is consistent with

data reported by Boncristiano *et al*, who showed that various VacA effects on Jurkat T cells could be blocked by NPPB, a non-specific chloride channel inhibitor (12). One of the VacA mutant toxins in the current study, VacA-Δ(6-27), potently blocked the effects of wild-type VacA on T cells (Fig. 27). This dominant-negative mutant VacA protein interacts with wild-type toxin, resulting in the formation of non-functional mixed-oligomeric structures (100). Inhibition of wild-type VacA activity by a dominant negative mutant protein is consistent with a model in which the formation of oligomeric VacA structures, such as membrane channels, is required for VacA-induced effects on T cells. We hypothesize that formation of anion-selective VacA membrane channels induces membrane-depolarization of T cells, and that this phenomenon is mechanistically important in the inhibition of IL-2-dependent T cell proliferation. Consistent with this view, Boncristiano *et al* recently reported that VacA could block ionophore-stimulated influx of calcium into T cells (12). Furthermore, a previous study demonstrated that activation of glycine-gated chloride channels induces membrane depolarization of T cells, resulting in a decreased open probability of plasma membrane calcium channels and in the inhibition of IL-2-dependent proliferation without affecting IL-2 secretion or NFAT activation (84).

The current results, along with two recent studies, provide evidence that VacA interferes with T cells proliferation by affecting multiple mechanisms. VacA is capable of inhibiting T cell activation by blocking the activation of NFAT (12, 34), and we now show that VacA is also capable of inhibiting IL-2-driven proliferation of T cells. In addition to these effects, VacA has been reported to interfere with T cell activation through a channel-independent mechanism that involves activation of intracellular

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signaling through the mitogen-actived protein kinases MKK3/6 and p38 and the Rac-specific nucleotide exchange factor, Vav (12). Together, these findings suggest that the effects of VacA on the immune system are likely to be multi-factorial and more complex than initially thought.

Gastric biopsies from *H. pylori*-infected individuals consistently demonstrate infiltration of immune cells including CD4$^+$ T helper cells, and specific anti-*H. pylori* T cells have been detected in the gastric mucosa (2, 22, 41, 45). Nevertheless, *H. pylori* is able to evade the immune response and establish persistent infection. Experiments in mice indicate that the quality of the T$_h$ cell response is critically important for eradication of *H. pylori* and prevention of *H. pylori*-induced pathology (2, 45). We propose that VacA might inhibit the clonal expansion and thus the acquisition of effector functions of infiltrating T$_h$ cells that have already been activated by *H. pylori* antigens. This immunosuppressive activity of VacA is likely to play an important role in the process by which *H. pylori* evades the adaptive immune response.

CHAPTER V

SUMMARY

Conclusions

H. pylori is the causative agent of most cases of duodenal ulcer disease and is considered a major risk factor for the development of gastric cancer. Experimental and epidemiological studies suggest that the vacuolating cytotoxin (VacA) is an important virulence factor in *H. pylori* pathogenesis, and that VacA contributes to the development of stomach and duodenal ulcers. Thus, functional and structural studies of VacA might lead to the development of VacA inhibitors that could be used to block VacA activities.

Approximately sixteen years have passed since the production of an *H. pylori* vacuolating toxin was first described by Leunk *et al* (43). Since then, VacA has been the focus of research of many laboratories around the world. In comparison with other bacterial toxins (*e.g.* cholera, diphtheria, and anthrax toxin), relatively little is known about how VacA exhibits many of its effects on mammalian cells. Over the past decade, several lines of evidence have indicated that VacA proteins form large oligomeric structures of ~1,000 kDa in mass. These VacA oligomers formed in solution have a flower-like structure and are composed of anywhere from 6 to 14 VacA monomers. The central ring in this VacA flower-like structure has been hypothesized to represent the VacA membrane channel, a hypothesis supported by the visualization of similar flower-like structures in cell membranes and the formation of VacA channels in planar lipid

bilayers. Thus, VacA-VacA interactions play an important role in VacA cytotoxic activity.

One major limitation in the VacA field has been the lack of recombinant over-expression systems that allow the production of large amounts of VacA proteins. Several groups have previously attempted to express recombinant VacA, but most of them have failed, or were able to produce only inactive recombinant proteins. Currently, VacA is purified from *H. pylori* broth culture supernatant in small amounts, sufficient to do limited experiments, but insufficient for undertaking structure-function analysis of this toxin. Today, the three dimensional structure of VacA is not known, and a comparison of the VacA amino acid sequence with proteins in the data base has not produced any insight into putative VacA domains.

Prior to undertaking this thesis projects, two putative VacA domains had been described in the literature. The mature secreted 88 kDa VacA can undergo limited proteolysis into 33- and 55-kDa fragments (p33 and p55 respectively), which might represent two VacA domains. However, very little was known about the function of these two domains. The main goal of this thesis was to characterize the role of p33 and p55 domains in the process of VacA oligomerization and intoxication of mammalian cells. Together, this thesis describes the functional characterization of the p33 and p55 VacA domains using a plethora of different approaches, in which each technique has provided insight into the importance of these domains for VacA assembly into oligomeric structures and cytotoxic activity.

Chapter II of this thesis describes the use of a yeast two-hybrid system to study possible physical interactions between the p33 and p55 VacA domains. It was

demonstrated that the p33 and p55 domains were able to form heterotypic complexes inside yeast cells. The utility of the yeast two-hybrid system to study VacA-VacA interactions was further demonstrated by analyzing a collection of VacA mutant proteins. Analysis of these mutant proteins indicated a strong correlation between the capacity of p33 and p55 proteins to interact, the formation of oligomeric structures, and vacuolating activity of full-length VacA proteins harboring the same mutations. In addition, analysis of a variety of p33 and p55 mutants and truncated proteins in the yeast two-hybrid system allowed for the mapping of putative minimal interaction domains in both the p33 and p55 domains. Additional data resulted from the generation and characterization of a VacA protein containing a FLAG-enterokinase tag (*FLAG-vacA*). Characterization of the FLAG-VacA protein indicated that the p33 and p55 interactions occur within VacA oligomeric structures formed by the *H. pylori* VacA protein. Collectively, these experiments provided strong evidence that the p33 and p55 domains of the VacA protein interact with each other and that these interactions play an important role in VacA oligomerization and vacuolating activity.

Chapter III describes expression and functional characterization of the p33 and p55 VacA domains. This is the first time that recombinant expression of active p33 and p55 VacA domains in *E. coli* has been described. In addition, this chapter also describes the role of these domains in the process of VacA-mammalian cell interactions. Previously it was thought that the p55 domain was responsible for VacA binding to mammalian cells and that the p33 domain was responsible for membrane insertion and channel formation. Studies using the recombinant p33 and p55 domains indicate that both of these domains are able to interact with host cell membranes. Interestingly, it was

demonstrated that even though the p33 and p55 bound to cells independently, both of the domains were required for VacA internalization into host cells. Furthermore, studies in which p33 and p55 were added sequentially to cells suggested that p55 binding, but not p33 binding, is critical for cell vacuolation. One possibility is that p33 and p55 may bind to different receptors, and that only the receptor bound by p55 allows the toxin to be internalized by cells. What is clear from these studies is that VacA might be able to bind to different molecules on the cell surface, but the process of VacA binding and internalization seems to required p55/cell interactions. These results also provide some insight into the complexity of VacA-cell interactions, since based on these results, VacA is the first pore-forming toxin for which toxin activity can be reconstituted from two separate fragments. This is interesting because it might suggest that VacA structure, as well as the mechanism of VacA membrane-insertion, might be different from what has been described for all the other pore-forming toxins.

This thesis work extensively describes the importance of the p33 and p55 domains for the process of VacA intoxication of mammalian cells. In addition, it also describes several novel tools for VacA research that will be useful for further investigation of VacA structure and function. The yeast two-hybrid system is one of the first systems in which VacA-VacA interactions could be studied in a high-throughput assay. This will allow the mapping of specific residues or regions important for p33/p55 interactions, which then can be validated by incorporating the mutations either in the *H. pylori* system or in the recombinant system. Also, the recombinant expression of active p33 and p55 domains is a major step forward in our efforts to further understand VacA structure function relationships, since these fragments can be generated in larger amounts than the amount

of VacA currently generated in the *H. pylori* system. Furthermore, purification of these domains might facilitate the crystallization and structural analysis of VacA, since several previous crystallization attempts using the full-length VacA toxin have failed.

VacA is able to exhibit many different effects on mammalian cells. One area of research that has become very popular recently is the characterization of VacA effects on immune cells. In chapter IV, VacA effects on primary human CD4$^+$ T cells were investigated. From this study it is clear that VacA exhibits different effects on primary human T cells compared to transformed human T cell lines. VacA inhibition of Jurkat T cell proliferation has been attributed to its potent inhibitory effect on IL-2 production (34). IL-2 is required for primary T cells to proliferate *in vitro* and *in vivo* upon activation. Interestingly, Jurkat T cells do not require IL-2 or TCR/CD28 activation for their proliferation *in vitro*. Therefore, the significance of VacA effects on IL-2 production in Jurkat T cells is somewhat controversial. Similar to reports using Jurkat T cells, when primary human T cells are intoxicated with VacA, they are impaired in their ability to proliferate. In contrast to Jurkat T cells, VacA has only minimal effects on IL-2 secretion in these cells, an effect that appears to be biologically insignificant. This finding is not surprising given that Jurkat T cells and primary human T cells have been shown to differ substantially in the transcriptional regulation of genes and the activation of multiple transcription factors upon activation (37, 60). Further analysis of VacA-mediated inhibition of primary human T_h cell proliferation indicated that VacA inhibits primary human T_h by impairing the IL-2-induced cell-cycle progression, resulting in an inhibition of IL-2-driven proliferation. The precise biochemical mechanism by which VacA inhibits IL-2-driven signals is not known, but formation of membrane channels by

VacA seems to be required. This observation is important, not only for the understanding of VacA-mediated effects on T cells, but little is known about the role of membrane channels in cell cycle progression and proliferation. It might be possible to use VacA as a tool to further understand how an anion-selective membrane channel might control cell proliferation.

Future Directions

<u>Purification of the recombinant p33 and p55 VacA domains</u>

One of the most important areas to emphasize in the future is the expression, purification, and further analysis of the p33 and p55 domains. Currently the expression of these proteins has not been optimized to obtain the highest amount or the highest levels of functionally active protein, which might facilitate the purification process. VacA has a tendency to stick to everything, including purification resins. In the case of the p33His and p55His VacA proteins, most of the protein remains is soluble when expressed at relatively low levels, but almost all the attempts to purify these protein by applying the soluble lysates to a nickel column have failed. The reason for this result is not known, but it might be possible that the VacA proteins are associated with *E. coli* proteins or *E. coli* lipids, which in turn make the His-tag inaccessible. In the future it might be worth trying different tags or different expression conditions that could support binding and elution of the recombinant proteins from the purification columns.

<u>Identification of the p33 and p55 receptors</u>

Several putative VacA receptors have been described in the literature, but whether VacA has a specific receptor is controversial. Interestingly, the work described in chapter III indicates that both the p33 and p55 VacA domains are able to interact with cell surfaces and that they interact with cells at higher levels when both p33 and p55 are present. Thus, it would be interesting to investigate if these domains bind to different cell molecules when added individually compared to when added together. In particular,

investigation of the role of lipid rafts seems really interesting. Previously, lipid rafts have been shown to be required for VacA vacuolating cytotoxic activity (81), presumably due to their role in VacA binding and/or internalization. Whether p33 and p55 localize to lipid rafts when added individually or when added together to cells is not currently known. These studies might provide insight into cell surface molecules required for VacA internalization, and might help to clarify the specific role of lipid rafts in the VacA intoxication process.

Intracellular localization of the p33 and p55 VacA domains

VacA has been previously shown to localize within cells in late endocytic compartments as well as mitochondria, but whether both p33 and p55 VacA domains are required for VacA targeting to specific intracellular organelles is not currently known. The feasibility to detect the p33 and p55 domains inside cells by immunofluorescence might allow us to investigate further the trafficking of these two domains to different cell organelles. It could be possible that the p33 and p55 domains target several different sites depending on whether the domains are assembled as an 88 kDa complex or as single p33 and p55 molecules.

Cellular target proteins of the p33 and p55 VacA domains

Two previous studies have identified two putative VacA intracellular-interacting proteins. First, screening of a HeLa cell library with p55 revealed that this VacA domain interacts with a novel protein of 54 kDa (VacA interacting protein 54 kDa; VIP54). VIP54 distribution in cells shows a fibrous pattern typical of intermediate filaments, to

the distribution of vimentin, but the physiological significance of the p55/VIP54 interaction is not clear. Second, screening of a human gastric mucosa cDNA library with different VacA fragments indicated that a VacA fragment containing mainly the C-terminus of p33 domain interacts with RACK1, a receptor for activated protein kinase C (PKC), but again the functional significance of this interaction is unknown.

One possible problem with the previous yeast two hybrid analyses is that there is no evidence that the VacA fragments used in these previous studies are capable of exhibiting any activity. Therefore, it could be possible that by using VacA bait proteins known to exhibit activity when expressed within yeast cells, different results might be obtained, which in turn, might provide insight into putative VacA cellular targets. Chapter III described the generation of p33 and p55 yeast two hybrid vectors that express functional p33 and p55 proteins, which could form p33/p55 complexes. These proteins could be used to screen cDNA libraries of mammalian cells. Since HeLa and gastric cell libraries have already been screened, it might be interesting to screen libraries from immune cells, since VacA has been shown to affect several immunologic processes. To this end, a mouse splenocyte cDNA library compatible with the yeast two-hybrid system used in chapter III has been obtained and will be screened against the functional p33 and p55 domains used in chapter three. This screen might provide insight into putative cell proteins that facilitate VacA effects on mammalian cells, in particular, immune cells.

Biochemical mechanism of VacA-mediated inhibition of primary human T cell proliferation

As described in chapter IV, VacA inhibits primary human T cells by impairing the IL-2 signaling pathway, but the precise molecular mechanism used by VacA to achieve

this end is not known. The IL-2 signaling pathway starts by the upregulation of the expression of IL-2 and the high affinity IL-2 receptor alpha (CD25), producing an autocrine system. Previously we have shown VacA does not affect the secretion of IL-2 or the surface expression of CD25 in primary human CD4$^+$ T cells (Chapter IV). Once the IL-2 binds to the trimeric IL-2 receptor (i.e. IL-2 receptor is composed of α, β, and γ chains) a complex signaling cascade is initiated resulting in two major outcomes, cell survival and cell proliferation. Whether VacA affects the oligomerization of the IL-2 receptor or signals triggered by the IL-2/IL-2R complex by inserting into T cell membranes has not been analyzed yet. Preliminary studies indicate that VacA does not affect the formation of the IL-2R trimeric complex nor the initial membrane proximal signals. This preliminary conclusion arises from studies of the activation of downstream molecules upon IL-2 stimulation. Primary human T cells intoxicated with either VacA or control buffer were each able to activate a key signaling molecule, signal transducer and activator of transcription 5 (STAT-5) upon IL-2 stimulation (i.e. STAT-5 is phosphorylated by the Janus kinase-3). Activation of STAT-5 upon IL-2 stimulation suggests that the membrane proximal signals triggered by the IL-2/IL-2 receptor complex are intact upon VacA intoxication.

In order to gain insight into the signaling pathways blocked by VacA in T cells, a global protein expression analysis might be useful. For these experiments, primary human T cells can be intoxicated with VacA and total cellular proteins isolated. To permit a rapid and efficient assessment of effects of VacA on protein expression, lysates from VacA-treated cells and control cells can then be used for a high-throughput immunoblot analysis with a panel of ~1000 antibodies (PowerBlot analysis; Becton-

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Dickenson Biosciences Pharmingen). This panel of antibodies includes a large number of antibodies against molecules involved in signaling and cell cycle regulation, as well as other antibodies of interest. These results might help to identify specific signaling pathways that are altered in response to VacA. In addition, transcriptional analyses of intoxicated cells might be useful.

VacA as a tool to understand signals involved in HIV infection of primary human T cells

Human $CD4^+$ T cells are the major target of HIV infection and replication *in vivo*. It has been well established that HIV infection of primary human T cells requires T cell activation signals, such as from the T cell receptor (TCR) and the co-stimulatory receptor CD28 (64). How T cell activation signals promote susceptibility of resting human T cells to HIV infection remains poorly understood, but the thresholds of T cell activation and IL-2 signals are critical for HIV to establish productive infection. Chapter IV described studies indicating that activation-induced proliferation of T cells can be inhibited by intoxication of these cells with VacA. VacA inhibits activation-induced proliferation of T cells without causing any detectable alterations in cell viability, and seems to affect the IL-2/IL-2 receptor proliferation signals. Therefore, VacA might serve as a new tool for further dissecting the T cell signaling pathways that are required for HIV infection of T cells. In fact, our preliminary studies suggest that VacA is able to inhibit HIV infection of T cells. Thus, investigation of the mechanism by which VacA inhibits HIV infection of T cells are likely to provide important new insights into our understanding of the T cell activation signals that are required for HIV infection, and may also reveal a very interesting pathogen-host-pathogen interaction.

<u>Last remarks</u>

How virulence factors produced by pathogens affect cellular processes is an important question in the microbiology field. One of the most interesting groups of virulence factors are bacterial toxins. In the case of *H. pylori*, the only known secreted toxin is the vacuolating cytotoxin (VacA). In comparison to other pore-forming toxins, VacA exhibits several unique properties. Most pore-forming toxins are extremely toxic toward their target cells, whereas VacA either does not cause cell death or induces apoptosis via a slow process. Another unique future of VacA is that it is internalized into mammalian cells, a phenomenon that is not observed with most other pore-forming toxins. Furthermore, VacA seems to exhibit a wide variety of effects on different mammalian cell types. All these observations suggest that VacA is a multifunctional toxin with unique properties. The work presented in this thesis provides insight into the structure-function complexity of this toxin.

One of the most interesting VacA activities is its immunosuppressive effect on human T cells. This VacA-mediated effect might play a role in the ability of *H. pylori* to establish a persistent infection that can last the life span of an individual. Understanding the mechanism by which VacA affects T cells might provide new insights into T cell signaling pathways. Moreover, modulation of T cells by VacA or related molecules might be clinically useful to suppress inflammatory diseases.

In summary, understanding of VacA mechanism of actions will not only be useful to understand the role of this toxin in *H. pylori* pathogenesis but it could also provide important information at the cell biology level that can then be used to address other important biological problems.

APPENDIX

List of publications

Torres, V. J., M. S. McClain, and T. L. Cover. 2004. Interactions between p-33 and p-55 domains of the *Helicobacter pylori* vacuolating cytotoxin (VacA). J Biol Chem **279:**2324-31.

Sundrud, M. S.*, V. J. Torres*, D. Unutmaz, and T. L. Cover. 2004. Inhibition of primary human T cell proliferation by *Helicobacter pylori* vacuolating toxin (VacA) is independent of VacA effects on IL-2 secretion. Proc Natl Acad Sci U S A **101:**7727-32. * Contributed equally to authorship

Torres, V. J., S. Ivie, M. S. McClain, and T. L. Cover. Functional properties of the p33 and p55 domains of the *Helicobacter pylori* vacuolating cytotoxin. (Manuscript in preparation)

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