Max-von-Pettenkofer Institute for Hygiene and Medical Microbiology
der Ludwig-Maximilian University München
Vorstand: Prof. Dr. Dr. Jürgen Heesemann

Studies on the function of the Cag Type IV Secretion System of *Helicobacter pylori* with integrin β1

Thesis Submitted for a Doctoral degree in Human Biology
At the Faculty of Medicine Ludwig-Maximilians-University,
Munich, Germany

Submitted by

Luisa Fernanda Jiménez Soto

from

Santafé de Bogotá, D.C.

2009

With approval of the Medical Faculty

Of the University of Munich

Supervisor/Examiner:	Prof. Dr. Rainer Haas
Second Examiner:	Prof. Dr. Georg Enders
Co-Examiners:	Priv. Doz. Dr. Thomas P. Hüttl
	Prof. Dr. Jochen Abb
Co- Supervisor:	Priv. Doz. Dr. Wolfgang Fischer
Dean:	Prof. Dr. med. Dr. h. c. M. Reiser, FACR, FRCR
Date of Oral Examination:	28.05.2009

Part of the work here presented will be/are published under the titles:

Fischer, W., Karnholz, A., Jimenez-Soto, L.F., and Haas, R. (2008) Type IV secretion systems in *Helicobacter pylori.* In: *Helicobacter pylori*: Molecular Genetics and Cellular Biology; Y.Yamaoka, Ed., Caister Academic Press, Norfolk, UK; p. 115-136

Jimenez-Soto, L.F, Sewald, X., Kutter, S., Ertl, C., Fischer, W., and Haas, R. (2008) RGD-independent interactions of the Cag Type IV Secretion System with integrin β1. Cell Host. Microbe. *Submitted*

Jimenez-Soto, L.F., Fischer, W., and Haas, R. (2009) Mechanism for the IL-8 induction via Cag Type IV secretion system from *Helicobacter pylori*. (*In preparation)*

Other publications published during the time of this thesis are:

Sherer, N.M., Lehmann, M.J., Jimenez-Soto, L.F., Horensavitz, C., Pypaert, M., and Mothes, W. (2007). Retroviruses can establish filopodial bridges for efficient cell-to-cell transmission. Nat. Cell Biol. *9*, 310-315.

Sherer, N.M., Lehmann, M.J., Jimenez-Soto, L.F., Ingmundson, A., Horner, S.M., Cicchetti, G., Allen, P.G., Pypaert, M., Cunningham, J.M., and Mothes, W. (2003). Visualization of retroviral replication in living cells reveals budding into multivesicular bodies. Traffic. *4*, 785-801.

Contents

Figure Index

Summary

The discovery of the association of *Helicobacter pylori* with gastric diseases 25 years ago opened a new view on the development of gastric disease and its relationship to microorganisms. With almost 50% of the human population having *H. pylori* in their stomachs, it is important to understand the real mechanisms by which the bacterium is able to cause gastric diseases such as gastritis, peptic ulcers and gastric cancer. Until now, several host as well as pathogenicity factors have been linked to the development of disease. One of them is the presence of the CagA protein and the Cag Type IV Secretion system (T4SS).

The Cag T4SS is encoded by 27 to 30 genes in a pathogenicity island. Several of these genes show homology to the prototype T4SS found in *Agrobacterium tumefaciens*, the *virB* system. Upon contact with the host cells, the Cag proteins assemble in a pilus-like structure.Through this structure, bacteria are able to translocate CagA, the only known effector protein, into eukaryotic cells. Once inside the eukaryotic cells, CagA interferes with several cellular processes, including cytoskeletal organization and kinase activity. The activity of CagA depends greatly on its tyrosine phosphorylation, catalyzed by several Src-kinase family members. Besides CagA translocation, the Cag apparatus itself is able to induce the production of chemokines from epithelial cells, such as IL-8, which are relevant for the immune response. Therefore, it is important to understand the precise mechanism of interaction of the Cag T4SS with the host cell, which was the purpose of this work.

In a first important observation, neutrophil-like cells, differentiated HL-60 cells (dHL60), were disturbed in their motility towards a chemoattractant *in vitro*. Using 2-dimensional (under agarose gel) as well as 3-dimensional (collagen matrix) conditions, the motility defect was found to be dependent on the presence of the CagA protein of *H. pylori*. Despite this activity, CagA showed a very low translocation into dHL-60 cells. In the search for the cause of this behaviour, it was discovered that integrin β1 levels on these cells varied in comparison to the parental HL-60 cell line, which showed normal CagA translocation levels. Using murine β1 integrin knockout cell lines, it was found that integrin β1 is essential for CagA translocation, acting as a receptor for the Cag T4SS. The presence of integrin β1 on the cell's surface could be correlated with the level of CagA translocation. Additionally, it was discovered that the interaction does neither require the cytoplasmic part of the integrin β1, nor its cytoplasmic signaling through the integrin-linked kinase (ILK). The interaction between

the Cag apparatus and the integrin β1 was confirmed to be direct, without involving extracellular matrix proteins as bridging elements.

From all 27 to 30 components of the Cag apparatus, three proteins showed a direct interaction with the integrin β1: CagA, CagY and CagI. In preliminary studies, the exact domain on the integrin β1 interacting with CagY has been limited to the PSI domain. Equally, the EGF and the β-tail domain (βTD) interact with CagA and CagI. Additionally, a specific antibody that limits the ability of integrin β1 to bend and extend (9EG7), eliminates translocation of CagA into the eukaryotic cell, suggesting that a switch between active and inactive conformations of integrin β1 is essential for CagA translocation. Furthermore, interference with lipid raft formation in the eukaryotic cell membrane and the clathrin pathway abrogated CagA translocation. In contrast, IL-8 induction, but not CagA translocation, required integrin β1 signaling via ILK. These data revealed the independence of both of these events, CagA translocation and IL-8 induction, tightly related with the Cag T4SS.

In order to get more insight into the structure of the Cag T4SS on the surface of *H. pylori,* cryo-electronmicroscopy studies have been started. Preliminary data show very regular pilus-like appendages on the bacterial surface, which still have to be verified as the Cag T4SS needles in future studies. The discovery of the integrin β1 as a receptor for the Cag apparatus and the involved cellular processes in the eukaryotic cell will provide more insight in the complicated but fascinating relationship between *H. pylori* and its host.

Zusammenfassung

Die Entdeckung der Assoziation von *Helicobacter pylori* mit Magenerkrankungen vor 25 Jahren hat eine neue Sichtweise auf die Entwicklung von Magenkrankheiten und seine Beziehung zu Mikroorganismen eröffnet. Fast 50% der menschlichen Bevölkerung ist mit *H. pylori* in ihren Mägen besiedelt. Daher ist es wichtig die wirklichen Mechanismen zu verstehen, durch welche das Bakterium Magenkrankheiten wie zum Beispiel Gastritis, Magengeschwüre und Magenkrebs verursachen kann. Bis jetzt wurden mehrere Wirts- sowie bakterielle Faktoren mit der Entwicklung der Krankheit verknüpft. Einer dieser Faktoren ist die Anwesenheit des CagA Proteins und des Cag Typ IV Sekretionssystems (T4SS).

Das Cag T4SS wird von 27 bis 30 Genen einer Pathogenitätsinsel kodiert. Einige dieser Gene zeigen Homologie zu einem prototypischen T4SS, dem *virB* System, welches in *Agrobacterium tumefaciens* gefunden wird. Nach Kontakt mit den Wirtzellen bilden die Cag Proteine eine Pilus-ähnliche Struktur. Durch diese Struktur, können die Bakterien das einzige bekannte Effektorprotein, CagA, in die Wirtszellen translozieren. In der eukaryotischen Zelle stört CagA mehrere zellulare Vorgänge, wie zum Beispiel die Zytoskellett-Organisation und die Aktivität der Src-Kinase. Die Aktivität von CagA hängt maßgeblich von seiner Tyrosinphosphorylierung ab, die durch mehrere Mitglieder der Src-Kinase-Familie katalysiert wird. Außer der CagA-Translokation kann auch der Cag-Apparat selbst die Produktion von Chemokinen, wie zum Beispiel IL-8, aus Epithelzellen induzieren, die relevant für die Immunantwort sind. Daher ist es wichtig, den präzisen Mechanismus der Wechselwirkung des Cag-T4SS mit der Wirtszelle zu verstehen.

In einer ersten wichtigen Beobachtung wurden Granulozyten-ähnliche Zellen, differenzierte HL-60 Zellen (dHL-60), in ihrer Beweglichkeit *in vitro* in Richtung von Chemoattraktanzien gestört. Unter 2-dimensionalen (Agarose-Gel) sowie 3-dimensionalen (Collagen-Gel) Matrix-Bedingungen konnte gezeigt werden, dass die Beeinträchtigung der Beweglichkeit von der Anwesenheit des CagA-Proteins abhängig war. Trotz dieser Aktivität zeigte CagA eine sehr niedrige Translokation in dHL-60 im Vergleich zu HL-60-Zellen. Dies konnte auf eine stark reduzierte Menge von Integrin β1 in diesen Zellen, im Vergleich zu der ursprünglichen HL60 Zelllinie, zurückgeführt werden.

Mit Hilfe von murinen Integrin β1-Knockout Zelllinien konnte gezeigt werden, dass Integrin β1 essentiell für die CagA-Translokation ist, indem es als Rezeptor für das Cag-T4SS dient. Die Anwesenheit von Integrin β1 auf der Oberfläche der Zelle konnte mit der Menge der

CagA-Translokation korreliert werden. Die Funktion des T4SS erfordert weder den zytoplasmatischen Teil des Integrins β1, noch seine Signaltransduktion durch die *Integrin-linked kinase* (ILK). Die Wechselwirkung zwischen dem Cag-Apparat und dem Integrin β1 konnten als direkt und unabhängig von Matrixproteinen bestätigt werden.

Von allen 30 Genprodukten des Cag-Apparates zeigten drei Proteine eine direkte Wechselwirkung mit dem Integrin β1: CagA, CagY und CagI. In vorläufigen Studien konnte die PSI Domäne auf dem Integrin β1 für die Wechselwirkung mit CagY identifiziert werden, während die EGF- und die β-tail Domäne (β-TD) mit CagA und CagI interagiert. Ein Integrin β1-spezifischer Antikörper (9EG7), der die Fähigkeit von Integrin β1 zur Konformationsänderung einschränkt, verhindert die Translokation von CagA in die eukaryotische Zelle, was nahelegt, dass ein Umschalten zwischen dem aktiven und dem inaktiven Zustand des Integrins β1 entscheidend für die CagA-Translokation ist. Weiterhin wurde durch die Zerstörung der *Lipid rafts* in der eukaryotischen Zellmembran und des Clathrin-abhängigen Aufnahmewegs die CagA-Translokation unterbunden. In Gegensatz zur CagA-Translokation erfordert die IL-8 Induktion eine Integrin β1 Signaltransduktion über ILK. Diese Daten offenbaren die Unabhängigkeit dieser beiden Ereignisse, der CagA-Translokation und der IL-8 Induktion, die beide mit dem Cag T4SS assoziiert sind.

Um mehr Einblick in die Struktur des Cag-T4SS auf der Oberfläche von *H. pylori* zu erhalten, wurden Kryo-Elektronenmikroskopie-Studien begonnen. Vorläufige Daten zeigen sehr regelmäßige Pilus-ähnliche Anhänge auf der bakteriellen Oberfläche, die dem Cag-T4SS entsprechen könnten. Die in dieser Arbeit beschriebene Entdeckung des Integrins β1 als ein Rezeptor für den Cag-Apparat und die beteiligten zellulären Prozesse in der eukaryontischen Zelle führen zu einen besseren Einblick in die komplizierte aber faszinierende Beziehung zwischen *H. pylori* und seinem Wirt.

1 Introduction

Helicobacter pylori (*H. pylori*) is a bacterium associated with gastric diseases, and it is considered to be the cause of gastric cancer. To get to know the bacteria better, it is necessary to know in which environment it is able to thrive and survive. This introduction will describe the biology of the stomach and associated pathologies to explain further the bacterium and the outcome of its presence in the stomach in more detail.

1.1 The Host's biology

H. pylori grows in one of the most aggressive environments inside the body, with so many mechanical stresses and low pH conditions (pH 2-5) that even the human body had to learn to protect itself from it by the creation of mucus (Allen and Garner, 1980). The stomach is generally divided into three sections: Fundus, Corpus and Antrum. The stomach's connection to the esophagus is limited by the Cardia and to the duodenum (first part of the intestine) by the Pyloric Valve (Figure 1-1). Each section has a distinct histological conformation. The fundus and corpus present parietal cells, which are responsible for the production of acid in form of hydrochloric acid (HCl); D cells produce somatostatin, and ECL cells which produce histamine. The antrum contains D-cells and ECL cells but no parietal cells. Instead, it contains G-cells, which produce gastrin.

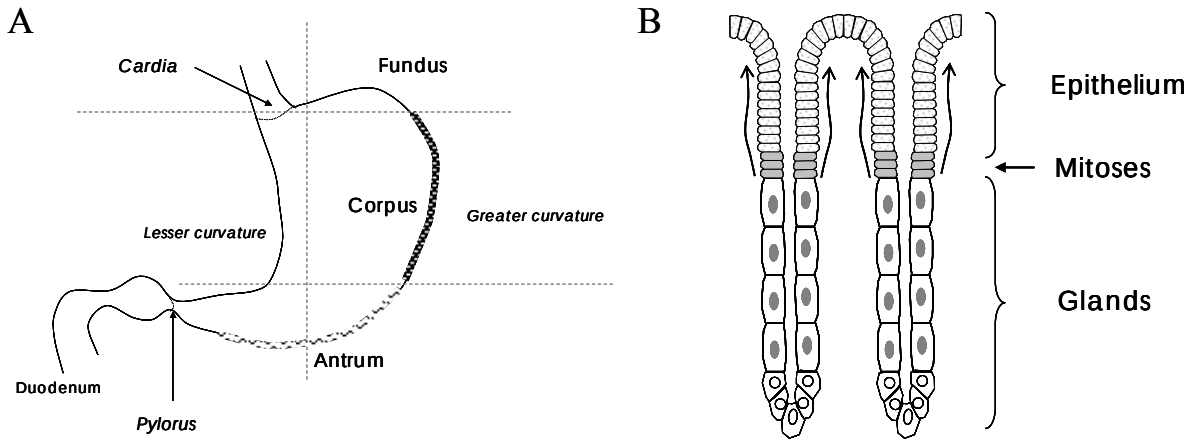


Figure 1-1 Stomach sections and histological distribution in the stomach mucosa

(A) Parts in which the stomach is anatomically divided. (B) Schematic distribution of the cells in the mucosal epithelia (Adapted from Croft,LN 1967)

The physiological function of the stomach depends on its ability to coordinate the secretion of acid in the gastric juice for digestion, and its regulation for the protection of the mucosa. It

can be described as a coordinated hormonal system. In the Antrum, G-cells detect stimuli from neural origin or of paracrine origin. As a response, gastrin is secreted and released into the bloodstream to find its destination: the ECL cells, parietal cells and D cells. Gastrin induces directly acid secretion by binding to the parietal cells through its receptor. The same effect can gastrin achieve indirectly by binding to ECL cells. The ECL cells secrete histamine as response to gastrin. Histamine binds through the H3-receptors on the parietal cells in order to induce the acid secretion. By gastrin binding to D cells in combination with histamine, it stimulates the opposite effect through the stimulation of somatostatin secretion. Somatostatin reduces the gastrin release and, at the same time, the acid release by the parietal cells (Joseph et al., 2003). All this activity in the stomach environment influences the behavior and development of *H. pylori* related gastritis, which shall be explored later on.

1.2 General changes in stomach biology associated with *Helicobacter pylori'* presence

The stomach physiology can be altered by the presence of *H. pylori*, but this is not the rule. To date there has not been a satisfactory explanation to the fact that, even though a high percentage of people have the bacterium in their stomachs, only a small proportion develops the pathologies that are normally associated with the bacterium's presence. It is known that bacteria can reside in both major regions of the stomach: the antrum, which is in the majority of the cases, or in the corpus region, which is in a small minority (Cave, 2001). While colonization of antrum is not often related to disease, colonization of the corpus is, however, associated with chronic inflammation of the gastric mucosa. Chronic inflammation can develop in atrophic gastritis, defined by a loss of gastric glandular cells, which can turn into an intestinal metaplasia. Intestinal metaplasia is defined by changes in the gastric epithelia that results in resemblance to intestinal epithelia. If untreated, these changes can further develop into gastric cancer (De Vries and Kuipers, 2007).

Coordination of the hormonal environment is essential for the stomach function and, in presence of *H. pylori,* several changes seem to be constant. Important changes are the excess of gastrin in plasma, the hypochlorhydria in antrum in initial infections, and the hyperchlorhydria in chronic infections. These effects on the acid regulation as well as in the hormonal equilibrium of the stomach might be the previous steps that drive the histological changes mentioned above.

1.3 *Helicobacter pylori*

1.3.1 History

Each disease has a story of discovery, but in the case of gastritis it is an adventure. For many years gastritis related diseases were not well understood and were linked to stress or nutrition disorders that caused swelling in the stomach lining and, if not treated, ulceration or stomach cancer.

Looking back to the first reports of gastric diseases, several causes were related to the presence of ulcers. Some early 1900s case reports described some gastric diseases, as the stomach syphilis that included sightings of spirochetes in the biopsies of removed stomach tissue from patients with ulcers (Graham, 1922). However, there were problems to confirm these sightings and they were therefore uncommon in the literature. Until the 1960s, case reports can be found where bacilli or bacteria absence in the extracted tissue of ulcers were dismissed by problems in the extraction and conservation of tissue material. Nevertheless, there was no doubt that the diseases of gastritis and gastric cancer were associated with the presence of a bacterium and its treatments included injections of heavy metal (including mercury) and 14 million units of penicillin (Madding et al., 1964). Even though this treatment was accepted as standard therapy, the difficulties to confirm the presence of bacteria gave way to other views shortly after. These new views opposed the idea of infectious agents being the cause of disease, dismissing it and considering environmental factors as the only cause of gastric diseases. Within these environmental factors, alcohol consumption, genetic predisposition (since it presented itself often in members of the same family) and consumption of acetylsalicylic acid were counted. Only one notion seemed to remain constant within these two ways of thinking: the discovery of a relationship between the presence of atrophic gastritis and pernicious anemia and its development to intestinal metaplasia as fore step of cancer development. However, in the second half of the 60's, an atrophic gastritis as consequence of an infective agent was considered as "medical curios" (Croft, 1967).

It took approximately 20 years, the combination of two Australian medical doctors, Barry J. Marshal and J.E. Warren, and an Easter holiday to discover a bacterium from biopsy material from a patient presenting gastritis. This bacterium seemed related to *Campylobacter spp.*, since it grew in the same conditions. It was described as a small spirochete with only two spirals and up to five polar flagella, making it differed from *Campylobacter spp.*, which

presenta a typical single polar flagella (Marshall and Warren, 1984). A name was given as *Campylobacter pylori* or Pyloric Campylobacter (PC), today known as *Helicobacter pylori*.

Nevertheless, the old dispute was still not settled and in 1985, in order to prove their theory right, the connection between disease and bacteria, Barry J. Marshall decided to apply the Koch postulates himself. He drank a 10 ml solution of bacteria and after 10 days of infection, colonization of the stomach was proven and related to the development of gastritis (Marshall et al., 1985) confirming for the first time a link between the presence of *H. pylori* and the gastritis symptoms. With their discovery, they finally resolved a "medical curious" into a fact and they brought back the old point of view seen in the beginning of the 1900's: a microorganism could be the cause of gastric cancer. Years later the World Health Organization (WHO) declared *H. pylori* as a Carcinogenic Type I, classifying a microorganism as cause of cancer. Their work did not only change the view of gastritis related maladies. They opened a possibility for many other diseases, whose exact cause have not yet been found. Because of their work and as recognition of Marshall and Warren's contribution to science, they were awarded the Nobel Prize of Physiology and Medicine in 2005.

1.3.2 Microbiological aspects of *Helicobacter pylori*

In 1986 *Campylobacter pylori* was described as a gram-negative curved bacillus, which initially was grown on Brain Heart Infusion (BHI) media with an atmosphere containing 10% CO_2 at 37°C during 3 to 6 days. The initial metabolic studies revealed a bacterium catalase- and oxidase-positive, with a 50% chance of growth at 42°C and urease positive. As for antibiotic sensitivity, it was described to resist to penicillin, erythromycin, tetracycline, gentamycin and cephalothin; and partial resistance to rifampicin and tinidazole (Marshall and Warren, 1984). Today it is considered a different genus based on 16S rRNA sequencing (Olsen GY, 1994), and it is given the name of *Helicobacter pylori* (*H. pylori*). More than 18 species of *Helicobacter spp.* are found in the stomachs or intestine of mammals. In some mammals, gastric diseases similar to those caused by *H. pylori* in humans are present, as it is the case for *Helicobacter felis* in mice and *Helicobacter mustelae* in ferrets (Owen, 1998).

1.3.3 Epidemiology: Tendencies, transmission and eradication

H. pylori has been present within the human population since the original migration of humans from East Africa more than 58000 years ago (Falush et al., 2003; Linz et al., 2007). Today it is estimated to be present in 50% of the human population (Blaser, 1993), with stronger presence in Africa (90%), America (50%) and Asian populations than within European countries (20%). Because of that close and old relationship, anthropological studies to confirm the evolution history of the human populations and its migrations from Africa to the rest of the world, are using the genetic information of *H. pylori* and its variation between strains to uncover the answers behind their theory (de Thé, 2007).

H. pylori's presence in the human stomach, as said before, can cause gastric ulcers and increase the risk of gastric cancer (Suerbaum and Michetti, 2002), which is today the 4th most common cancer in the United States (Greenlee et al., 2000). To explore the possibility of a prophylaxis management of *H. pylori* infections, several studies have evaluated the benefit in preventive eradication of the bacterium even in cases where no sign of disease is present. These studies hoped to diminishing future health costs. From these studies, no conclusive results were obtained. These results have somehow been expected by some scientist, as Martin J. Blaser. He considers *H. pylori* part of our normal flora and proposes that *H. pylori* have evolved with humans. In his opinion, *H. pylori* eradication should only be necessary when disease appears (Blaser, 1997). Some truth could be in there. In recent reports, there have been cases were esophageal pathologies have appeared once the bacterium has been eradicated from the stomach. These reports have established a negative correlation between *H. pylori* presence and esophagus diseases, as if the presence of *H. pylori* would protect from them (McColl et al., 2008). Additionally to these reports, there are several indications of not only the bacteria's presence being the cause of disease. There are as well dietary factors like high consumption of processed carbohydrates (Rocco and Nardone, 2007), genetic polymorphism in the host (Algood and Cover, 2006) and patterns in physical activity (Cheng et al., 2000).

The relationship between presence of the bacteria and disease is not the only aspect of *H. pylori* that remains to be defined. *H. pylori* ways of transmission are surrounded by the same veil of unknowns. While various publications state that infection with *H. pylori* occurs early in the childhood and propose a horizontal transmission from the parents to the child or from siblings (de Thé, 2007). Others define a fecal-oral or oral-oral transmission. Some even define *H. pylori* as water borne pathogen (Engstrand, 2001). Although these studies are

central for transmission prevention, no real correlation has been made yet. With no way to prevent its transmission, the only possibility left is the eradication by the use of antibiotics.
The eradication treatment recommended today is called the PPI triple / quadruple therapy defined by the use of a Proton Pump Inhibitor (PPI), omeprazole, in combination with a set of antibiotics as metronidazole, clarithromicin and/or tetracycline. The constant emergence of antibiotic resistance in combination with low compliance of the therapy by the patient makes the eradication difficult and not very successful (Megraud, 2007). New antibiotics and alternative eradication therapies are being investigated (Kihira et al., 1996) because of the association of diseases like MALT lymphoma with the persistence of *H. pylori* infections.

Another approach that could shed light on the development of disease associated with *H. pylori* is the understanding of the bacteria, their molecular mechanisms that leads to survival within the human population, as well as their pathogenesis and virulence factors.

1.3.4 Pathogenesis and virulence factors

As biological organism, *H. pylori* requires an environment where it can survive and thrive. This environment is the human stomach. It will be easy to think that it has evolved to live there in order to avoid competition with other bacteria. After all, very few bacterial species are reported to establish a niche in the stomach. Nevertheless, this cannot exclude that the stomach could have proven to be a good place to enter the body's inner tissues. Whatever the bacteria's reasons, *H. pylori* has developed survival techniques to deal with the physical conditions found here and to deal with the immune system. These survival techniques are known as virulence and pathogenicity factors.
Urease is one of the first survival measures that *H. pylori* applies to conquer the stomach physical topography. Urease is an enzyme capable of converting the acid environment (~ pH 2) around the bacteria to more basic and tolerable surroundings through hydrolysis of urea into carbon dioxide and ammonia (Ferrero et al., 1992). Urease has been recently associated as well with a role in the evasion of the immune system. In this case, as interaction with macrophages occurs, the enzyme seems to confer the bacterium the ability of survival by formation of megasomes and inhibiting phagocytosis (Schwartz and Allen, 2006). Another mechanism of survival is *H. pylori*'s flagella. In *H. pylori*, expression and assembly of flagella have been predicted to involve more than 50 putative proteins of which the two subunits encoded by *flaA* and *flaB* genes have been extensively characterized (Tomb et al, 1997). During experiments carried out on gerbil stomachs, the polar flagella help the bacteria

to keep its ground amid the mucosa, overcoming the constant mucus secretion and maintaining a "territory" that ranges from 0 - 25 µm area of the mucus layer above the epithelia. The same studies established that the bacterium manages to orientate in the stomach mucosa using a movement towards certain pH gradients and carbonate concentrations (Schreiber et al., 2004; Schreiber et al., 2005). Movement towards a gradient can indicate tropism towards the epithelia in order to bind to it. In these experiments, only a small percentage of the bacteria are localized on the epithelia cells, being the majority in the mucosa layer *per se*.

This data uncovers a controversial and complex system required for colonization. In studies by Kavermann (Kavermann et al., 2003), genes were found to be required for a successful colonization. Schreiber et al confirmed the mutagenesis effects on these genes. Some of these data were not surprising as for genes encoding for motility components like the flagella associated proteins. The same results were expected from genes encoding for acid survival as the urease genes. However, the impact of other genes on colonization, as a collagenase protein or Outer Membrane Proteins (OMPs), was startling. Adhesion onto the epithelia cells is not a common observation made in gerbils during colonization. The necessity of adhesion related proteins for a successful colonization could be explained by the fact that adhesion seems to be triggered by changes in the tissue's pH and, in some conditions, it might lead to invasion (Schreiber, S; personal communication). If the observations made in the gerbil model are comparable to the situation in the human stomach, adhesion of *H. pylori* can be essential for the survival of the bacterium in the stomach under pH changing conditions.

In general, it is known that adhesion of *H. pylori* can be mediated by adhesins and OMPs present in it, for which 32 paralogous genes are predicted (Alm et al., 2000; Alm and Trust, 1999; Tomb et al., 1997). These OMPs are grouped in two families termed *hop* and *hor* genes. From these families the best-known members are BabA, SabA, AlpA, AlpB, and OipA. The BabA (Blood group antigen-binding adhesin) binds to fucosylated Lewis B (Le^b) blood group antigen found in MUC5A on gastric cells (Aspholm-Hurtig et al., 2004; Ilver et al., 1998). SabA (Sialic acid-binding adhesin) binds to Lewis X (Le^x) antigens present in inflamed tissue (Mahdavi et al., 2002), neutrophils (Unemo et al., 2005) and erythrocytes (Aspholm et al., 2006). OipA (Outer inflammatory protein) plays an important role as adhesion/colonization factor. AlpA, and AlpB (Adherence-associated lipoprotein) are essential colonization factors presumably binding to receptors on the cells surface (Odenbreit et al., 1999).

With the adhesion onto the epithelial cells, the thin line between colonization and invasion can be easily crossed. The invasion of the tissue could indicate the progress of disease. Therefore, factors for evasion or modulation of the immune response are necessary.

Within these factors, it can be named the HP-NAP (*H. pylori* Neutrophil-associated Protein) which has been described as an oligomeric protein consisting of 10-12 monomers of a 17-kDa polypeptide with homology to iron proteins (Tonello et al., 1999). HP-NAP is able to induce neutrophils to produce oxygen radicals (Satin et al., 2000), and it has been described to act as an adhesin (Namavar et al., 1998; Teneberg et al., 1997).

VacA toxin (Vacuolating Cytotoxin A) and the Type IV Secretion Systems (T4SS), Cag and ComB, are as well part of the virulence factors. VacA is an AB type toxin encoded by the *vacA* gene. It presents a precursor of ~140 kDa, which is processed to an approximately 95-kDa protein that is release in the media. Bybeing a secreted soluble toxin, VacA has the remarkable capacity of reaching areas of the stomach mucosa that bacteria cannot reach. In this way, its immuno-modulatory effect can be successfully achieved. By disrupting the phosphatase activity of Calcineurin, VacA prevents the translocation of NFAT into the nucleus and therefore inhibits the IL-2 synthesis and secretion by T-cells. By doing so, it inhibits T-cell proliferation (Gebert et al., 2003). This effect on proliferation occurs after VacA binds to a receptor on T-cells, the integrin β2 subunit (Sewald et al., 2008). Another phenotype related to VacA activity is the formation of vacuoles expressing markers for late endosomes (Rab7) and lysosomes (Lamp1). This property has been linked with the disruption of the normal proteolytic activity of the endocytic pathway, giving an advantage to the bacterium in case of phagocytosis (Montecucco et al., 2001). Additionally, VacA has the ability to increase the permeability of polarized epithelia, thereby possibly increasing the supplement of nutrients available to the bacterium in the mucosa (Pelicic et al., 1999).

Two T4SS in *H. pylori* are to date associated with pathogenicity, the ComB and the Cag systems. Their relevance for pathogenicity involves different aspects. The ComB system allows the bacterium to take up DNA from the outside and integrating it in the genome, giving the bacteria great versatility for adaptations (Hofreuter et al., 2001). In the case of the Cag system, its relationship to pathogenicity has been extrapolated from epidemiological data. These data show a higher incidence of gastric tissue pathologies in cases where its effector protein, CagA, is present. Further description of both T4SSs and some of their cellular effects will follow.

1.3.4.1 Type IV secretion systems

To date, there are six types of secretion systems described. They are used by gram-negative bacteria to transport cargo proteins across the inner and outer membranes. The best described secretion systems are the Type III Secretions Systems (T3SS) and its injectisome (Mueller et al., 2008) and the Type IV Secretions Systems (T4SS) because of their common association with pathogenicity.

T4SS are common transporters used by pathogenic bacteria. The first of them described was the VirB system from *Agrobacterium tumefaciens*, which contains at least 12 genes involved in the transfer of the Ti plasmid into the host plant cell causing modifications that give origin to the Crown Gall disease. With the discovery of the VirB system 30 years ago, a translocation system was identified by which an interkindom DNA transfer was possible. Furthermore, with the identification and sequencing of its components, new secretion systems began to be discovered in other pathogenic Gram-negative bacteria by sequence homology. Today they are found in several bacteria, e.g. *Bordetella pertussis* (Ptl System), *Brucella spp.* (VirB system), *Bartonella henselae* (Trw System and VirB-homologue (vbh)), *Legionella pneumophila* (Dot/Icm system, Lvh system (Bandyopadhyay et al., 2007)) and *Helicobacter pylori* (ComB and Cag system) (Fischer et al., 2002). The purpose of translocating proteins across membranes works in different ways. Some of them do not require host cell contact, releasing the effector protein to the outside, as is the case of *B. pertussis* and its pertussis toxin. Pertussis toxin is assembled in the bacteria and exported by the T4SS into the extracellular milieu to find its target cells. The other systems seem to need a bactera cell- host cell contact to deliver the effector proteins into the target cell, as it occurs for *H. pylori* (Cag system), *Legionella* and *Rickettsia* T4SS (Fischer et al., 2002).

In the case of *Helicobacter pylori*, the ComB and the Cag systems are representatives of a Type IV Secretion System (T4SS). Recently it has been described a third T4SS in *H. pylori*, the TFS3, whose function remains to be established (Kersulyte et al., 2003).

1.3.4.1.1 ComB system

The ComB system was identified as a Type IV Secretion apparatus based on sequence homologies with the VirB system of *Agrobacterium tumefaciens*. It contains the ComB2 to ComB4 and ComB6 to ComB10 as core proteins. Other proteins that are necessary for transformation, but are not part of the T4SS are RecA, DprA and ComH (Hofreuter et al., 1998; Karnholz et al., 2006; Smeets and Kusters, 2002).

The ComB system is responsible for natural competence of *H. pylori*. Nedenskov-Sorensen et al. discovered the uptake of free DNA by *H. pylori* in 1990. The process can be divided into the binding of DNA to the surface of the bacterium, the transport of the DNA through the periplasm and the degradation of one strand leaving a single strand DNA molecule. This single strand DNA is translocated through the inner membrane to the cytoplasm where it is finally incorporated in the chromosome (Dubnau, 1999). The fact that almost all strains contain the ComB system, leads to contemplate the possibility that it plays an important role in the survival of the bacteria and not only an advantage for genetic diversity. Transposon mutagenesis studies (Kavermann et al., 2003) revealed the relevance of the ComB system under *in vivo* situations, in which ComB4 was essential for colonization in the gerbil model. However, the reason for the relevance *in vivo* remains to be elucidated.

1.3.4.1.2 Cag Type IV Secretion System

The 27 to 30 ORFs (Open Reading Frames), found in the Cag pathogenicity island, encode the Cag Type IV secretion system. Figure 1-2 illustrates the genes found on the strain 26695 *cag*PAI.

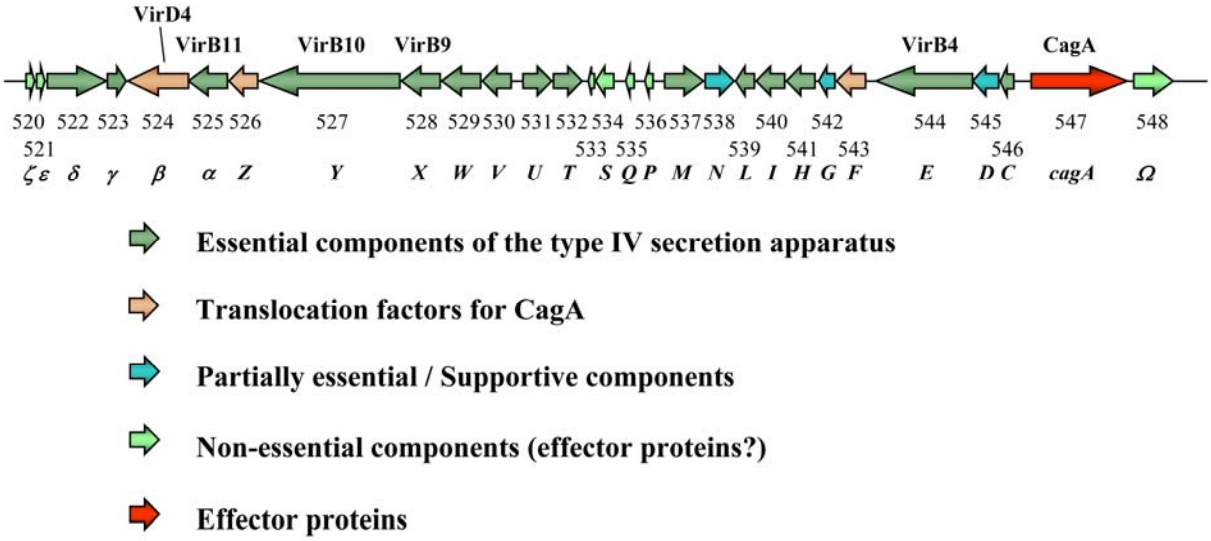


Figure 1-2 Cag Pathogenicity Island genes

Genes encoded in the Cag pathogenicity island and its homologous genes in the VirB system. Three denominations systems for the genes contained in the T4SS are used to date, of which only two are explained here. Number of the ORF based on the sequence of *H. pylori* 26659 strain and a single letter code for the genes' names.

Associated with the functionality of the secretion system is the capability of inducing chemokine secretion, such as Interleukin 8 (IL-8) and RANTES, upon interaction with the eukaryotic cell (Odenbreit and Haas, 2002). Equally important is the ability to translocate the

CagA (Cytotoxic Associated Gene A) into the eukaryotic cell (Odenbreit et al., 2000). A systematic mutagenesis study of the genes encoded by the *cag* PAI was performed (Fischer et al., 2001), in which phenotypic effects of the Cag apparatus, IL-8 induction and CagA phosphorylation (CagA-P) were analyzed, giving an insight in the functionality of the genes encoded. They have been classified in five groups: proteins essential for the apparatus functionality, support or accessory proteins, proteins essential for CagA translocation, effector proteins and proteins of unknown function.

As effector protein, only CagA, encoded by gene *hp0547*, has been identified. Based on the IL-8 induction ability, 14 proteins are classified as essential proteins for the secretion apparatus. The proteins encoded by *hp0526, hp0524* (*virD4* homologue), *hp0543* and *hp0540* were shown to be essential only for CagA translocation; while *hp0520*, *hp0521*, *hp0534*, *hp0535* and *hp0536* are not necessary neither for IL-8 induction nor for CagA translocation (Fischer et al., 2001).

From the proteins essential for the functionality of the Cag apparatus are worth to mention the CagY (homologue to VirB10), CagT (homologue to VirB7), and CagX (homologue to VirB9). All have been defined as structural proteins in electron microscopy studies (Rohde et al., 2003; Tanaka et al., 2003). In the case of CagY, studies of its composition unveiled remarkable characteristics. Its homology region to VirB10 proteins is present in the C-terminal part of the protein. Additionally, CagY contains two repeat regions. Repeat Region I (RRI) corresponds to the N-terminal part, while Repeat Region II (RRII) is localized in the middle region of the protein. Both repeat regions show no homology to any known bacterial protein. Between RRI and RRII, a transmembrane domain has been predicted. The same prediction has been made for the region found between the RRII and the VirB10 homology region (Liu et al., 1999). Structural studies have found other proteins related to the apparatus structure. This is the case for CagT (HP0532), which shares homology with the VirB7, a structural protein of the VirB system (Cascales and Christie, 2003). Using antibodies and electron microscopy studies, Sasakawa et al. were able to localize this protein on pilus-like extensions found only on bacteria with an intact Cag island. The same observation was made with the VirB9 homologue, CagX (HP0528) (Tanaka et al., 2003). Eventhough Sasakawa's studies could not detect CagY on the surface of these structures, Rohde et al. could confirm the presence of this protein along of pilus-like structures with concentrated areas around their tips, as well as areas at the bacteria's surface (Rohde et al., 2003).

The current working model of the Cag apparatus is pictured in figure 1-3. Most of the interactions between the proteins shown here have been elucidated using Yeast-2-Hybrid

(Y2H) assays complemented by biochemical assays, which have confirmed the interactions in independent ways (Kutter et al., 2008; Pattis et al., 2007).

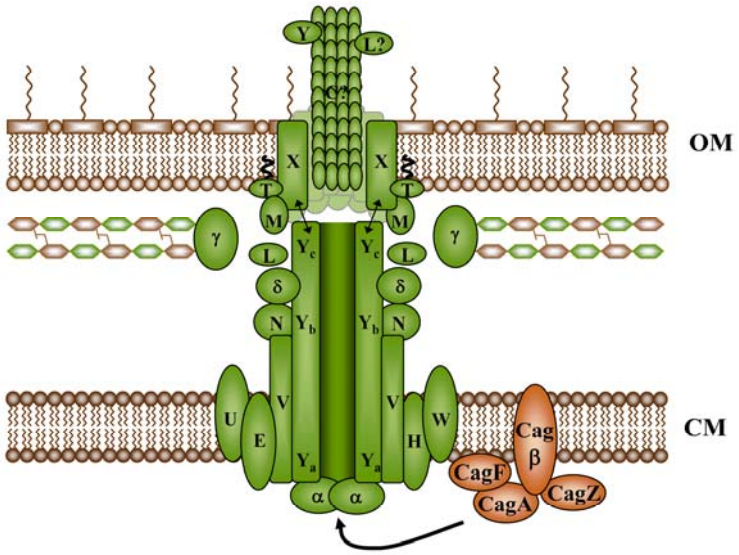


Figure 1-3 Model for the CagPAI apparatus

Actual working model of the Cag apparatus based on defined protein interactions and functionality.

CagA is the only effector protein yet known to be translocated by the T4SS into the target cell. Once inside the cell's cytoplasm, it is phosphorylated by Src and Abl family kinases on its EPIYA motifs. The EPIYA motifs are located in the C-terminal region of the protein (Poppe et al., 2007; Selbach et al., 2002). The variations in these phosphorylation motifs determine the biological activity of the CagA. A closer look at these motifs revealed a difference between CagA+ Western Strains (Western CagA-Specific Sequence, WSS) and Eastern Strains (East-Asian CagA-Specific Sequence, ESS), explaining the variability in disease progression between infections in East Asia and Western cases (Higashi et al., 2002). The five amino acid sequence constitutes the EPIYA motif, which is recognized by cellular kinases that phosphorylate the tyrosine (Y) residue. Based on their adjacent amino acid sequences, the EPIYA motifs have been classified in four types: A, B, C and D. The Eastern Strains contain the combination ABD while Western Strains present ABC EPIYA motifs (Hatakeyama, 2006).

However, not only the variations on its EPIYA motifs determine the effects of CagA on the target cell. CagA's presence inside the cell and its effects vary as well depending of its phosphorylation status. Non-phosphorylated CagA has been associated with changes in transcription patterns through direct binding to E-Cadherin, which disrupts the Cadherin/β-catenin complex, translocating β-catenin into the nucleus. Once β-catenin is translocated in

the nucleus, it upregulates pathways like the *cdx1* pathway, which regulates intestinal differentiation (Murata-Kamiya et al., 2007). Through the same interaction, CagA disrupts tight junctions of polarized epithelia and induces invasiveness, as seen in studies with polarized MDCK cells (Bagnoli et al., 2005).

In case of phosphorylated CagA, its functions include the dephosphorylation of cellular proteins and the inactivation of kinases essential for the normal operation of the cell. The dephosphorylating activity has been linked with the Shp-2 protein. Shp-2 phosphatase activity is stimulated by the direct binding of phosphorylated CagA to the phosphatase. (Higashi et al., 2002). Additionally, it has been reported that the dephosphorylation is reinforced by the inhibition of Src-kinase by phosphorylated CagA, which results in dephosphorylation of Cortactin (Selbach et al., 2003). Cortactin dephosphorylation induces some of the cytoskeleton modifications associated with the phosphorylated form of CagA. Another way of altering the cytoskeleton is through Crk-dependent activation of the Arp2/3 complex (Suzuki et al., 2005) and the dephosphorylation of Focal Adhesion Kinase (FAK) by Shp-2 (Tsutsumi et al., 2006). The cytoskeleton modifications are visible in two phenotypes presented by infected cells: “hummingbird” and cell scattering. The “hummingbird” phenotype is presented by AGS cells (Adenogastric carcinoma cells, human) as an elongation of the cells shape (Segal et al., 1996). Cell scattering occurs after infection of cells that have been serum starved previously, increasing the movement of cells. This phenotype has been associated with the presence of the Cag apparatus as well (Moese et al., 2004).

1.4 Aim of the studies

The Type IV Secretion System (T4SS) encoded on the *cag*PAI and its effector protein CagA represents one of the most intriguing virulence mechanisms of *H. pylori*. In the course of this thesis I will try to identify the molecular mechanisms implicated in the interaction of the Cag apparatus with the host cell. I will describe the elements from the bacteria necessary that make possible the transfer and translocation of the effector protein CagA. The main objectives of these studies are the identification of the receptor for the Cag apparatus on the cell's membrane and the signaling necessary to make this interaction a successful one for CagA translocation.

2 Materials and Methods

2.1 Materials

2.1.1 Cell lines

3051/9	Human Adenogastric carcinoma cell line
AGS	Human Adenogastric carcinoma cell line (ATCC CRL-1739)
Caco-2	Human Colon carcinoma cell line
CHO-K1	Chinese Ovary Hamster cell line Clone K1
CHO-K1 β1A	Chinese Ovary Hamster cell line Clone K1, hITGB1A
CHO-K1 β1com	Chinese Ovary Hamster cell line Clone K1, hITGB1 com
CHO-K1 β1TR	Chinese Ovary Hamster cell line Clone K1, hITGB1A transmembrane and extracellular domains
GD25	Murine Fibroblast-like embryonic cell line, ITGB1 -/-
GD25β	Murine Fibroblast-like embryonic cell line, mITGB1 in trans
GD25β 1A	Murine Fibroblast-like embryonic cell line, ITGB1 +/+
GE11	Murine Epithelia-like embryonic cell line, ITGB1 -/-
GE11β	Murine Epithelia-like embryonic cell line, hITGB1 in trans
H10	Mouse breast epithelial cell line, cMet -/-
H10 cMet	Mouse breast epithelial cell line, cMet -/-, human cMet in trans
HEK-293	Human Embryonic Kidney Cell line
HeLa	Human Cervical carcinoma cell line (ATCC 57)
HL-60	Human promyelocytic leukemia cell line
J774A.1	Murine Macrophage cell line (ATCC 170)
Jurkat EG-1	Human T-Cell line (ATCC TIB-152)
Kato III	Human epithelia cell line

MDCK	Marby Darby Canine Kidney cell line (ATCC CCL-34)
MKN45	Human Adenogastric carcinoma cell line
T47D	Human Breast Carcinoma cell line
T47D Met	Human Breast carcinoma cell line, over expressing human cMet receptor
β-2-4	Murine Fibroblast-like embryonic cell line, ITGB1 -/-
β-2-4-8	Murine Fibroblast-like embryonic cell line, mITGB1 +/+

2.1.2 Bacteria Strains

2.1.2.1 Escherichia coli strains

DH5α	F^-,ϕ80*lacZ*ΔM15Δ(*lacZYA-argF*)U169, *deoR*, *recA* 1, *endA* 1, *hsdR* 17(r_K-,m_k+), *supE* 44, *thi* -1, λ^- *gyrA* 96, *relA* 1 (GIBCO,BRL)

2.1.2.2 Helicobacter pylori strains

P12	Clinical Isolate from the Department of Medicine Microbiology and Immunology, University of Hamburg
P217	Clinical isolate.
P145	ATCC 45526
26659	Genome Sequenced Wild strain (Tomb et al., 1997)
LJ-H1	P12Δ547 Kan^R
LJ-H56	P12Δ544 Cam^R
LJ-H54	P12Δ527 Cam^R
LJ-H17	P12ΔPAI, Kan^R
LJ-H12	P12Δ547 pHel4 GFP, Kan^R, Cam^R
LJ-H10	P12 Wild type pHel4 GFP, Kan^R, Cam^R

LJ-H15	P12ΔPAI pHel4 GFP, Kan^R, Cam^R
LJ-H53	P12ΔCagA ::CagA-GSK, Kan^R, Cam^R

2.1.3 Plasmids

pLJ-2	pGEX-4T3 containing CagYc (*H. PYLORI*0527c)
pJP109	pGEX-4T-3 containing CagY RRII (*H. PYLORI*0527b)
pJP110	pGEX-4T-3 containing CagY RRI (*H. PYLORI*0527a)
pHel4 GFP	pHel4 containing GFP protein for expression in *H. pylori*.

2.1.4 Oligonucleotides

LJ-10	GGTGTCGTGTTTGTGAATGCT
	Sense primer for detection of ITGB1 mouse
LJ-11	GGCAACTTCTCCCTGCTTTC
	Antisense primer for detection of ITGB1 mouse
LJ-12	GCAGAATCCAAAGTAAATGTCCTG
	Sense primer. Recognizes Human ITGB1 Variant 1A
LJ-13	GATTAGAAGAGGTGATAGAAAGCAC
	Antisense primer. Recognizes Human ITGB1 Variant 1A
LJ-14	GACAGTTCTTCTTCTTGCCA
	Sense primer. Recognizes Human ITGB1 Variant 1B
LJ-15	CCACAATCAAGTCATCCCAA
	Antisense primer. Recognizes Human ITGB1 Variant 1B
LJ-16	GCTGGAATTGTTCTTATTGGCCTT
	Sense primer. Recognizes Human ITGB1 Variant 1D
LJ-17	TTCACCGGCAATTTAGAGACCAG
	Antisense primer. Recognizes Human ITGB1 Variant 1D
LJ-20	AAAAAGCAGGCTCCGCCAATGTTCAAGAGAGCTGAAGAC

	Forward AttB1 sequence vWF domain integrin β1
LJ-21	AGAAAGCTGGGTCTAAATGACTTCTGAGGAAAG
	Reverse AttB1 sequence vWF domain integrin β1
LJ-22	AAAAAGCAGGCTCCGCCAATGTCTTACTGCAAGAACGGG
	Forward AttB1 sequence Laminin / EGF-like domain from integrin β1
LJ-23	AGAAAGCTGGGTCTATTTCCTGCAGTAAGCATC
	Reverse AttB1 sequence Laminin / EGF-like domain from integrin β1
LJ-24	AAAAAGCAGGCTCCGCCAATGAATTTACAACCAATTTTC
	Forward AttB1 sequence extracellular domain from integrin β1
LJ-25	AGAAAGCTGGGTCTAGATGTCTGGACCAGTGGG
	Reverse AttB1 sequence extracellular domain from integrin β1
LJ-38	CGTGGATCCATCATAGCTCTAGATAAACTCATAGGC
	Sense primer of *hp0527* introducing BamHI site
LJ-39	CGTAGCGGCCGCTTAATTGCCACCTTTGGGGCTTGTGGT
	Antisense primer of *hp0527* introducing NotI site
LJ-57	CGTGGATCCAATTTACAACCAATTTTCTGG
	Sense primer of PSI domain (ITGB1 Aa 1-99) introducing BamHI site
LJ-58	CGTAGCGGCCGCCTATCCTTTGCTACGGTTGGTTAC
	Antisense primer of PSI domain (ITGB1 Aa 1-99) introducing NotI site
LJ-59	CGTGGATCCTCAGAAGGAGTAACAATAAG
	Sense primer of EGF (1-4) domains (ITGB1 Aa 410-625) introducing BamHI site
LJ-60	CGTAGCGGCCGCCTAAAACTTCGGATCTGTACAC
	Antisense primer of EGF (1-4) domains (ITGB1 Aa 410-625)

	introducing NotI site
LJ-61	CGTGGATCCTGTCGTGTGTGTGAGTGCAACC
	Sense primer of β-tail domain (ITGB1 Aa 575-728) introducing BamHI site
LJ-62	CGTAGCGGCCGCCTAGATGTCTGGACCAGTGGGACAC
	Antisense primer of β-tail domain (ITGB1 Aa 575-728) introducing NotI site
LJ-63	CGTGAATCCGTAACCAACCGTAGCAAA
	Sense primer of I-like domain (ITGB1 Aa 96-460) introducing BamHI site
LJ-64	CGTAGCGGCCGCCTAATCCTTTTTTGGACACTTATTTC
	Antisense primer of I-like domain (ITGB1 Aa 96-460) introducing NotI site
dsRNA ILK	Sense strand 5'-GGAAGAGAUAGUUUGAUUUTT-3'
	Antisense strand 5'-AAAUCAAACUAUCUCUUCCTG-3'

2.1.5 Reagents and Solutions

2.1.5.1 Reagents

BBL Brucella Broth (BD Falcon), Fluoroprep (BioMeriux), Coomassie Brilliant Blue G250) (Biomol), Acrylamide/Methylenbisacrylamide 30% (29:1) and X-Gal(Roth), Streptomycin, Trimethoprim, Vancomycin, Nystatin, Ampicillyn, Phrobol-12-myristat-13-acetate PMA, Cytochalasin D, Bafilomycin A1, Genistein, Cycloheximide, 5-Brom-3-chlor-3-indolylphosphate-p-toluidinsalt BCIP, Ionomycin, Guanidine-HCL, Ethidium Bromide, Leupeptin, Sodium-ortho-orthovanadatee, Paraformaldehyde, Glutaraldehyde, Pepstatin, Triton X-100, Tween 20, DMSO, Dansylcadaverine, Methyl-β Cyclodextrin (Heptakis), ammonium chloride (Sigma-Aldrich); Chloramphenicol (Serva), Kanamycin, Phenylmethylsulfonylfluorid PMSF (Merck), GC Agar, LB Agar, LB broth (Oxoid), Sephadex-G-50, Glutatione Sepharose (Pharmacia), Zinc staining kit, Silver staining kit , Precision Plus Protein Standards All Blue (BioRad); U73211 (Calbiochem); Dynasore was a

kind gift from Dr. Tomas Kirchhausen.

2.1.5.2 Solutions and buffers

PBS 10X	2 g/l KCL, 80 g/l NaCl, 2 g/l KH_2PO_4; 14,4 g/l Na_2HPO_4 (29 g/l Na_2HPO_4 . $12H_2O$). Autoclave if necessary and store at RT.
2X SDS loading buffer	100 mM Tris HCl pH 6,8; 4% SDS; 0,2% Bromophenol blue, 20% Glycerol, 10% β-Mercaptoethanol (optional)
5X SDS loading buffer	10% SDS; 0,5 M Tris HCl (pH 6,8); 50% Glycerol, 5% Bromophenol blue. Store at room temperature.
NBT detection buffer	0,1 M Tris HCl pH 9,6; 0,01% w/v NBT, 0,005% w/v BCIP, 7 mM $MgCl_2$
Agarose Loading buffer 6X	0,25% Bromophenolblue; 0,25% Xylene Cyanol FF, 30% Glycerol; in TAE buffer
50X TAE buffer	242 g/l Tris Base, 57,1 ml/l Glacial Acetic Acid, 50 mM EDTA
PFA 1X	Paraformaldehyde 4%, PBS (+Ca^{2+}, +Mg^{2+}), pH 7,4
PFA 10X	Paraformaldehyde 27%, PBS 10X, pH 7,4

2.1.6 Peptides, Proteins and enzymes

Proteins	Fetal Horse Serum (PAA Laboratories GmbH), Bovine Serum Albumin BSA (Sigma), Alkaline Phosphatase-Protein A (Sigma), purified integrin $\beta_1\alpha_5$ (VLA-5) and integrin $\beta_1\alpha_1$ (Chemicon), Collagen Type I from bovine skin (Vitrogen 100®, Nutacon, Holland).
Peptides	RGD (Gly-**Arg-Gly-Asp**-Ser-Pro-Lys) peptide, RAD (Gly-**Arg-Ala-Asp**-Ser-Pro-Lys) peptide (Sigma)
Enzymes	Thrombin (Calbiochem); trypsin (Pancreatic), fibronectin (human plasma) and proteinase K (Sigma); DNAse I and RNAse A (Roche).

2.1.7 Antibodies

2.1.7.1 Primary antibodies

α- Actin (H-196)	Mouse monoclonal antibody against actin from different species (Santa Cruz Technologies)
α- CagA (AK257)	Rabbit polyclonal antibody against the C-terminal part of CagA from *Helicobacter pylori*.
α- CagA (AK268)	Rabbit polyclonal antibody against the N-terminal part of CagA from *Helicobacter pylori*.
α- CagC (AK576)	Rabbit polyclonal antibody against the C-terminal part of CagC from *Helicobacter pylori.*
α- CagC (AK577)	Rabbit polyclonal antibody against the C-terminal part of CagC from *Helicobacter pylori*
α- CagY (AK273)	Rabbit polyclonal antibody against the Repeat Region II of CagY from *Helicobacter pylori*.
α- CagY (AK280)	Rabbit polyclonal antibody against the Repeat Region I of CagY from *Helicobacter pylori*.
α- CD18 PE	Mouse monoclonal antibody against human CD18 (integrin β2) Clone 6.7. R-Phycoerithrin (PE) conjugated. (BD Biosciences)
α- CD29 FITC	Hamster monoclonal antibody against extracellular part of ITGB1 from different species. Clone Ha2/5. FITC conjugated. (BD Biosciences)
α- Clathrin	Mouse monoclonal antibody against the heavy chain of clathrin (Pharmacia)
α- GST	Mouse monoclonal antibody against Glutatione S-Transferase (GST) protein (Sigma)
α- *H. pylori* (AK175)	Rabbit polyclonal antibody against a soluble extract of *Helicobacter pylori* P1 strain.

α- IL-8	Mouse monoclonal antibody against human IL-8 (BD Biosciences)
α- IL-8 biotin	Mouse monoclonal antibody against human IL-8, biotin conjugated (BD Biosciences)
α- ILK	Mouse monoclonal antibody against human integrin-linked kinase (ILK). (Sigma)
α- ITGB1	Rabbit monoclonal antibody against cytoplasmic tail of human integrin β1 (ITGB1). (Cat. Nr. AB1952 from Chemicon)
α- ITGB1 (Clone 3S3)	Mouse monoclonal antibody against human ITGB1 (Wilkins JA)
α- ITGB1 (Clone 4B7)	Mouse monoclonal antibody against human ITGB1. (Calbiochem)
α- ITGB1 (Clone 6S6)	Mouse monoclonal antibody against human ITGB1 (Wilkins JA)
α- ITGB1 (Clone 8E3)	Mouse monoclonal activating antibody against human ITGB1 (Mould et al., 1995)
α- ITGB1 (Clone 9EG7)	Rat monoclonal antibody against extracellular part of human and mouse ITGB1 (Calbiochem)
α- ITGB1 (Clone 12G10)	Mouse monoclonal inactivating antibody against human ITGB1 (Mould et al., 1995)
α- ITGB1 (Clone AIIB2)	Rat monoclonal antibody against extracellular part of ITGB1. Inactivating antibody. Prepared from hybridoma cells.
α- ITGB1 (Clone B3B11)	Mouse monoclonal antibody against human ITGB1 (Wilkins JA,1996)
α- ITGB1 (Clone B44)	Mouse monoclonal antibody against human ITGB1 (Wilkins JA,1996)
α- ITGB1 (Clone JB1A)	Mouse monoclonal antibody against human ITGB1 (Wilkins JA,1996)

α- ITGB1 (Clone K20)	Mouse monoclonal antibody against extracellular part of human ITGB1. (Santa Cruz Technologies)
α- ITGB1 (LM534)	Mouse monoclonal antibody against extracellular part of human ITGB1. (Chemicon)
α- ITGB1 (Clone N29)	Mouse monoclonal antibody against human ITGB1 (Wilkins JA,1996)
α- P-tyr (4G10)	Mouse monoclonal antibody against tyrosine phosphorylated proteins (Upstate)
α- P-tyr (PY99)	Mouse monoclonal antibody against tyrosine-phosphorylated proteins (Santa Cruz Technologies)
α- Tubulin	Mouse monoclonal antibody against human and mouse tubulin alpha subunit (Upstate)

2.1.7.2 Secondary antibodies

α- mouse IgG $Alexa_{488}$	$AlexaFluor_{488}$-conjugated goat monoclonal antibody (Molecular Probes)
α- mouse IgG $Alexa_{555}$	$AlexaFluor_{555}$-conjugated goat monoclonal antibody (Molecular Probes)
α- mouse IgG $Alexa_{567}$	$AlexaFluor_{567}$-conjugated goat monoclonal antibody (Molecular Probes)
α- mouse IgG AP	Alkaline phosphatase-conjugated goat monoclonal antibody (Sigma)
α- mouse IgG Pox	Peroxidase-conjugated goat monoclonal antibody (Sigma)
α- rabbit IgG $Alexa_{488}$	$AlexaFluor_{488}$-conjugated goat monoclonal antibody (Molecular Probes)
α- rabbit IgG $Alexa_{555}$	$AlexaFluor_{555}$-conjugated goat monoclonal antibody (Molecular Probes)
α- rabbit IgG Pox	Peroxidase-conjugated goat monoclonal antibody (Sigma)
α- rat IgG $Alexa_{488}$	$AlexaFluor_{488}$-conjugated goat monoclonal antibody

	(Molecular Probes)
α- rat IgG $Alexa_{555}$	$AlexaFluor_{555}$-conjugated goat monoclonal antibody (Molecular Probes)

2.1.8 Cell culture solutions and additives

For each cell line, a different medium is necessary Here is a general description of media and additives used in cell culture.

Cell Media	High Glucose DMEM, RPMI-1640, MEM, F12 and DMEM:F12. (Invitrogen/GIBCO BRL), 10X MEM
Antibiotic	Penicillin/Streptomycin and Gentamicin (Invitrogen/GIBCO BRL)
Selective	G418 (Geniticin) and Hygromicin B (PAA laboratories), Puromycin (SIGMA-Aldrich) and Zeocin (Invitrogen/GIBCO BRL).
Supplements	Fetal Calf Serum and L-Glutamine (Invitrogen/GIBCO BRL). Fetal Bovine Serum Superior (Biochrom), 7,5% Sodium Bicarbonate (GIBCO)
Others	Trypsin-EDTA (TE), Dulbecco PBS ($+Ca^{2+}$, $+Mg^{2+}$) and Dulbecco PBS ($-Ca^{2+}$, $-Mg^{2+}$) (Invitrogen/GIBCO BRL), DMSO and EDTA (SIGMA Aldrich).

2.1.9 Consumables and Equipment

2.1.9.1 Consumables

Sulphate modified latex fluorescent beads (Sigma), Carboxyl Magnetic beads (Chemicell), X-Ray film (Fuji, A.Hastenstein), Dialysis membranes (Medicell), Dialysis membranes for small volumes (Pierce), ELISA Maxisorp plates (Nunc), Cell scrappers (Falcon), FACS tubes (Becton Dickinson), Freezing Tubes 2 ml (Nalgene), PVDF membrane (Bio-Rad), High Range Protein Marker (Bio-Rad), Cell culture treated plates (Corning), 0,2μm- Sterile filters (Millipore), Cell culture treated bottles (75 cm^2 and 175 cm^2) (BD Falcon), Filter paper (Whatman), Cell culture inserts 3 μm pores (Corning). Paraffin pellets (Fluka).

2.1.9.2 Equipment

PAGE-Mini Gel System and Voltage Units Power Pac 300 and Power Pac 1000 (Bio-Rad), Incubator (Binder), Incubator *Ultima* (Revco), Microincubator MI22C (Scholzen), Geldocumentation System (Bio-Rad), Absorbance Reader Sunrise (TECAN systems), FACS EPICS ® XL-MCL (Coulter), Spectrophotometer DR/2000 (Hach), Multichannel Pipette (MATRIX Corporation), Agarose Gel Electrophoresis chamber (Bio-Rad), Centrifuge Biofuge 15R and Megafuge 3.0 R(Heraeus), Centrifuge Mikro 20 (Hettich), Developing Cassette for X-Ray films (Rego), Magnetic Stirrer MR 3001 (Heidolph), Medical Film Processor FPM-100A (Fuji film), Microscope DM IRB (Leica), Confocal Microscope (Leica) and TCS Software (Leica), PCR Thermocycler (ThermoHybaid), PCR Thermocycler Microcycler Personal (Eppendorf), Pipette Transferpette-8 (20-200µl and 0,5-10 µl)(Brand), Scales (Fischer Biotech), pH Meter (WTW), Semi-dry Blotting Chamber (Fischer Biotech), Sterile Hood (BDK), Vacuum Centrifuge Speed-Vac DNA 110 (Savant), Vortex Gene 2 (Scientific Industries), Water Bath (GFL).

2.2 Methods

The methods here described are the general protocol for the work with bacteria, DNA and cell culture as well as the methods developed and/or used in frame of this work.

2.2.1 Working with bacteria

2.2.1.1 Culture

Helicobacter pylori strains were passed from a 3-day culture after defrosting. Usually the bacteria would have grown on a GC agar plate for 24 hours before passed or used for experiments. The conditions of growth were at 37°C in an atmosphere composed of 85% N_2, 10% CO_2 and 5% O_2. In the case of growth in serum-free media, bacteria were grown minimum two passages before using them in the experiments.

For the growth of *Escherichia coli* strains, it was used LB-agar for the selection of transfomants, LB media for growth of bacteria producing fusion proteins and Terrific Broth (TB) for plasmid isolation. All growth media were complemented with their respective antibiotic and/or inducer.

GC Agar serum	36 g/l GC agar, 8% Horse Serum, 1% Vitamin mix, 1 mg/l Nystatin, 5 mg/l Trimethoprin, 10 mg/l Vancomycin
GC Agar serum-free	36 g/l GC agar, 1X Cholesterol (GIBCO), 1% Vitamin mix, 1 mg/l Nystatin, 5 mg/l Trimethoprin, 10 mg/l Vancomycin
LB Agar	32 g/l Lennox-L Agar
LB Media	20 g/l Lennox-L Medium
Vitamin Mix	100 g/l a D-Glucose, 10 g/l L-Glutamine, 26 g/l L-Cystein, 0,1 g/l Cocarboxylase, 20 mg/l Fe(III)-Nitrat, 3 mg/l Thiamin, 13 mg/l p-Aminobenzin acid, 250 mg/l Nicotinamid-adenine dinucleotide (NAD), 10 mg/l Vitamine B12, 1,1 g/l L-Cystine, 1 g/l Adenine, 30 mg/l Guanine, 0,15 g/l L-Arginine, 0,5% Uracil.
TB Solution 1	12 g/l Tryptone, 24 g/l Yeast Extract, 0,4% Glycerol
10X TB Solution 2	0,17 M KH_2PO_4; 0,72 M K_2HPO_4

Antibiotics were used in the following concentrations,

Ampicillyn	100 mg/l
Chloramphenicol	30 mg/l (LB Medium, TB medium and LB agar)
	6 mg/l (GC Agar)
Kanamycin	50 mg/l (LB Medium, TB medium and LB agar)
	8 mg/l (GC Agar)

2.2.1.2 Freezing of E.coli

From an overnight liquid-grown culture, 350 µl from the culture were mixed with 350 µl of a sterile 70% (v/v) Glycerol solution, and frozen directly by storage at -70°C.

2.2.1.3 Freezing of H. pylori

Bacteria grown on plates were resuspended in 1 ml Freezing Media for *H. pylori* and frozen directly by storage at -70°C.

Freezing Media *H. pylori*	10% FCS, 20% Glycerol, 70% Brucella Broth, sterile filtrated.

2.2.1.4 Transformation of chemical competent cells

For the transformation of *E.coli* bacteria, chemical competent cells were prepared after Hannahan's method. The prepared bacteria suspension was aliquoted in 50 µl aliquots and store at -70°C. Shortly before transformation, an aliquot was defrosted slowly in ice. Plasmid DNA (between 20 ng and 500 ng) was added to the bacteria and incubated for 30 minutes in ice. The bacteria were then exposed to 42°C for 45 seconds and immediately cooled in ice. After the temperature shock, 1 ml warm LB media was added to the bacteria and incubated for one hour at 37°C. The suspension was pulse-centrifuged , the supernatant discarded and the bacteria pellet resuspended in 50 µl LB media. The resuspended pellet wasplated on LB agar plates containing the antibiotic necessary for the selection of transformants.

2.2.1.5 Transformation of H. pylori

H. pylori bacteria were grown on GC serum plates overnight to form a very thin film, which was collected and resuspended in 1 ml Brucella Broth containing 1% FCS to a final OD_{550} 0,1. Bacteria were left for one hour at 37°C with 10% CO_2, after which DNA was added. After 4 hours of incubation at 37°C with 10% CO_2, bacteria were collected, pulse-centrifuged and resuspended in 50 µl Brucella broth. The suspension was plated onto GC agar plates containing the antibiotic in the concentrations needed.

2.2.1.6 Integrin $\beta_1\alpha_5$ staining of H. pylori

GFP expressing bacteria were grown on serum-free or serum complemented GC agar plates. For serum-free media, two passages were necessary to reduce the serum proteins still adherent on the bacteria. Thin grown bacteria were collected with a sterile cotton swab and resuspended in cold PBS (-Ca^{2+}, -Mg^{2+}). OD_{550} was measured and a 1 ml suspension OD_{550} 0,1 was created. In each well of a 24-well plate, cover glass slides were added. Per well 250µl of ice cold PBS (-Ca^{2+}, -Mg^{2+}) was added followed by 250 µl of the OD_{550} 0,1 bacteria suspension. The bacteria were adhered to the glass slide present on the wells through centrifugation for 10 minutes at 4000 rpm at 4°C. Not attached bacteria were removed and washed with 1 ml cold PBS (+Ca^{2+}, +Mg^{2+}). To each well 500 µl PBS (+Ca^{2+}, +Mg^{2+}) were added as well as 3 µl of fluorescent conjugated integrin$\beta_1\alpha_5$-$Alexa_{555}$. The protein and

bacteria were incubated in darkness one hour at 4°C. Unbound integrin and bacteria were removed with three washing steps using 500 µl of cold PBS . Finally, samples were fixed in 500 µl 1X PFA solution for one hour in darkness at 4°C. Cover glass slides were washed two times with PBS (-Ca^{2+}, -Mg^{2+}) and mounted on as glass slide containing a drop of mounting medium (Fluoprep™). Solidification of mounting media took place at 4°C overnight. After sealing of the cover slides with nail polish, they were stored in darkness at 4°C until imaging with a Leica Confocal microscope.

2.2.1.7 Binding of H. pylori to integrin $\beta_1\alpha_5$ coated beads

Thin growing bacteria on GC serum plates were collected in serum-free DMEM media, its absorbance at wavelength 550 (OD_{550}) was measured and suspensions of 1 ml in cold serum-free DMEM with a final OD_{550} 0,1 were done. To each solution, 10 µg of RGD containing peptide were added to inhibit unspecific binding of integrin to bacteria. After addition of 5 µl of coated magnetic beads, the solution was shortly vortexed and placed in a magnetic field for five minutes. To wash unspecific binding, three wash steps were done with 1 ml serum-free DMEM. After the third washing, beads were resuspended in 100 µl of serum-free DMEM. Serial dilutions were made and plated to count the amount of bacteria adhered to the beads. As control for the viability of the bacteria originally used, serial dilutions were as well made from the original OD_{550} 0,1 suspension. Plated samples grew at 37°C in an atmosphere composed of 85% N_2, 10% CO_2 and 5% O_2. After 3-4 days, colonies were counted. Three colonies from each strain were chosen to grow them for further binding assays to integrin $\beta_1\alpha_5$ coated beads.

2.2.1.8 Induction of Cag apparatus expression for Cryo-EM studies

Bacteria grown on serum GC agar plates were collected in PBS (-Ca^{2+}, -Mg^{2+}) and their OD_{550} estimated. Bacteria were then added to 250µl AGS cell exudates (see 2.2.3.18) to a final OD_{550} of 0,3 per ml in a 24-well and incubated for 60 to 105 minutes at 37°C with 5% CO_2. 10 µl of the suspension were carefully taken for EM studies, keeping the rest of the samples for biochemical studies.

2.2.2 General DNA work

2.2.2.1 DNA extraction

Plasmid DNA was prepared depending on their following use with two different methods: the method from Holmes and Quigley was used for rapid screening of transformants. Qiagen prep kits were used for isolation of plasmids that were to be used in PCR reactions or to transfect eukaryotic cells.

2.2.2.1.1 Isolation of plasmid DNA after Holmes and Quigley (Holmes and Quigley, 1981)

Suspected positive clones for selection were grown overnight on 1/6 of LB plates at 37°C, collected using a cotton swab and resuspended in 300 µl STET buffer. After adding 10 µl of a lysozyme solution, the samples were mixed gently by inversion and incubated in ice for no more than 5 minutes. To lysate the cells completely, cells were exposed one minute to 100°C. The cell debris, as well chromosomal DNA, was separated from the solution containing the plasmid DNA by centrifugation at 13000 rpm for 15 minutes. After removing the pellet with a sterile toothpick, 200 µl of isopropanol was added to the supernatant, mixed by inversion and incubated for 10 minutes at -20°C. The plasmid DNA was collected in a pellet after a 10-minute centrifugation at 13000 rpm. To eliminate any traces of isopropanol, awashind step with 500 µl of a 70% Ethanol solution followed. The solution was centrifuged at 13000 rpm for 1 minute and all the ethanol removed. After drying the DNA pellet, it was resuspended in distilled water or TE buffer.

STET	8% Sucrose, 5% Triton X-100, 50 mM EDTA, 50 mM Tris HCl pH 8,0.
Lysozyme solution	10 mg/ml lysozyme in STET buffer.

2.2.2.1.2 QIAGEN preps

For the preparation of plasmid DNA, transformed bacteria grew for 16-20 hours at 37°C in TB media under constant shaking. TB components were sterile mixed shortly before inoculation in a proportion 1:10 (Solution 2: Solution 1). After growth, bacteria were centrifuged at 4000 rpm for 20 minutes at 4°C, and the supernatant discarded. Extraction of DNA was achieved using the mini (midi or maxi) prep kit from QIAGEN with some modifications. For 250 ml cultures, the Maxi-prep kit (QIAGEN) was used. The bacterial

culture was centrifuged at 4000 rpm for 20 minutes at 4°C. The supernatant was carefully discarded and the pellet resuspended in 10 ml buffer P1. Lysis was achieved adding 10 ml of buffer P2. The lysis reaction was neutralized with 13 ml of buffer N3. To separate bacterial debris from DNA suspension, the solution was centrifuged at 4000 rpm for 40 minutes followed by the collection of supernatant. The supernatant was added onto a pre-equilibrated column with QBT buffer. After the supernant has runned through the column, the column was washed three times with 15 ml of wash buffer. To eluate the DNA from the column, 2 ml of Elution buffer was added onto the column and collected.. DNA concentrations were estimated at OD_{260}.

P1 Buffer	50 mM Tris HCl pH 8, 10 mM EDTA, 100 µg/ml RNAse
P2 Buffer	200 mM NaOH, 1% SDS
N3 Buffer	3 M Potassium Acetate. Adjust pH with Glacial Acetic Acid to pH 5,5
QBT Buffer	750 mM NaCl, 50 mM MOPS pH 7, 15% Isopropanol, 0,15% Triton X-100
Wash Buffer	1 M NaCl, 50 mM MOPS pH 7, 15 % v/v Isopropanol
Elution buffer	Tris 10 mM pH 8, 10 mM EDTA

2.2.2.2 DNA purification and concentration

Plasmid DNA that was used for transfection experiments was concentrated and purified using phenol:chlorophorm. The solution containing plasmid DNA was mixed in a proportion 1:1 with phenol:chlorophorm solution (1:1) and vortexed (only for plasmids smaller than 10 kb). After centrifugation at 13000 rpm for 10 minutes at room temperature, the upper phase was collected and mixed with 3M sodium acetate (1/10 Volume). 9-Volumes Ethanol 100% were added and then mix by inversion. The resulting solution was centrifuged at 13000 rpm, 4°C for one minute and the supernatant discarded. The pellet was washed once with 500 µl ethanol 90%, centrifuged again at 13000 rpm for one minute, and the ethanol discarded. The DNA pellet was dried and resuspended in 50 µl TE buffer. The DNA concentration was estimated and the DNA solution was stored at -20°C until needed.

2.2.3 Work with cell culture

In general, cells grew in the optimal media and physical conditions described in previous publications or recommended by the ATCC (American Type Culture Collection). If not explained otherwise, all cells were splitted once they reached a maximum of 90% confluency.

2.2.3.1 Cell synchronization

Cells that were used for synchronization experiments were splitted using the PBS/EDTA method (see 2.2.3.2). For synchronization, 70% confluent cells were washed once with serum-containing media (complete media, CM). The CM was removed and cell culture media lacking serum (serum-free media) was added. Cells were incubated at 37°C with 5% CO_2 overnight. In the case that cells showed extreme susceptibility to this treatment, they were synchronized using media containing 0,1% serum for 18 hours.

2.2.3.2 PBS /EDTA suspension of adherent cells

To avoid extra stress on the cells or the lost of a possible receptor / interaction partner from the surface of the cells, adherent cells were splitted using a PBS (-Ca^{2+}, -Mg^{2+}) solution with 2 mM EDTA (sterile filtrated). For a 75 cm^2 bottle, cells were washed once with 10 ml of PBS (-Ca^{2+}, -Mg^{2+}), and after removal of the PBS (-Ca^{2+}, -Mg^{2+}), 3 ml of the PBS/EDTA solution was added. Cells were incubated for 10 minutes or until detachment of the cells and mixed with 13 ml cell media, which stopped the EDTA effect on the cells. Cells were finally collected by centrifugation for further work.

PBS/EDTA PBS (-Ca^{2+}, -Mg^{2+}), 2 mM EDTA

2.2.3.3 Estimation of viable cells

Using cells in solution, a dilution 1:10 was done in Trypan Blue solution (10 µl cell suspension, 90 µl Trypan blue) and 10 µl were loaded onto a Neubauer Chamber. Only cells in the chambers external quadrants that possessed no blue color were counted. The total number of cells counted was divided by 4, multiplied by the dilution factor and by the chamber's factor (10^4) to obtain the final amount of viable cells per milliliter of suspension.

2.2.3.4 Fixation of cells using the "in flagrante" method

Because of the fast fixation effect observed using this protocol, it has being named "in flagrante" method. Using a 10X Paraformaldehyde solution pre-warmed at 37°C, 1/10th of the solution was added to the samples to be fixed. Incubation for 20 minutes at room temperature followed. Cells were then washed with PBS (-Ca^{2+}, -Mg^{2+}) once and samples stored in a 1% PFA solution at 4°C until immunostaining.

2.2.3.5 General protocol for the detection of proteins on cell's surface using flow cytometry

Adherent cells were detached from wells using the PBS/ EDTA method and collected by centrifugation. For non-adherent cells, they were collected and centrifuged at maximum 300*g* for 5 minutes and the media removed. After collecting cells, pellets were resuspended gently using PBS (-Ca^{2+},-Mg^{2+}) and washed two times to remove any traces from EDTA in the solution. After counting and resuspending cells to a concentration 1x10^6 cells/ml, the detection followed. In the case of detection using fluorescent-labeled primary antibodies, the manufacturer's recommended concentrations were used and incubation followed for one hour at 4°C. If the use of a non-labeled primary antibody was necessary, it was diluted 1:1000 and incubated one hour at 4°C or 20°C. After washing three times, a secondary fluorescent-labeled antibody was added using a 1:1000 dilution followed by one-hour incubation at 4°C. Two wash steps with PBS removed the unbound antibody. After the cells were fluorescent labeled, they were resuspended in 1 ml of PBS or FACS buffer and stored in ice and darkness until measurement.

2.2.3.6 FACS quantification of integrin β1 levels on the membrane

AGS cells were splitted in 60 mm plates using the PBS/EDTA method in a dilution 1:4 calculating that 48 hours later they will be 100% confluent. After approx. 30 hours, cells were synchronized overnight. One well was fixed using the "in flagrante" method (see 2.2.3.4). This well represents the initial amount of integrin β1 on the surface after the synchronization. Media containing serum was added, and at the following time points, the same fixation method was used: t = 0 (2min), 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 and 120 minutes. Each

fixation was for one hour at 4°C store before the addition of antibody. Cells were blocked with an IP blocking buffer overnight at 4°C. Next day cells were washed two times with PBS ($+Ca^{2+}$, $+Mg^{2+}$) and supernatant containing primary antibody AIIB2 was added in a concentration 1:100 in blocking buffer for minimum 6 hours at 4°C. Antibody was removed and washed twice with PBS, leaving the reaction in PBS overnight at 4°C for increased specificity. Next day, the washing step was repeated four times, and $Alexa_{488}$ conjugated anti-Rat antibody was added in a concentration of 1:2500 for one hour at 4°C. Excess antibody was removed with four washing steps and cells were scrapped from the plate's surface in 1 ml PBS. The cells were collected and measure their fluorescence intensity in an EPICS® XL-MCL cell sorter (Coulter). Data obtained was analyzed using WinMDI software. By using the mean fluorescence from the population, curves were graphed that describe the amount of integrin β1 present on exposed side of the membrane at the time of fixation in a time dependent way.

Blocking buffer 10% FCS (v/v) in PBS ($+Ca^{2+}$, $+Mg^{2+}$).

2.2.3.7 General protocol for Immunostaining

After the fixation of the samples by the "*in flagrante*" method (see 2.2.3.4), samples on glass coverslides were blocked overnight using 500 µl sterile blocking solution at 4°C. To remove the blocking solution, coverslides were washed three times with 1 ml PBS, each time for 10 minutes. 250 µl of the blocking buffer with primary antibody (1:1000) were added for one hour at 37°C. To remove unbound antibody, five washing steps were repeated. After the primary antibody was removed, 250 µl of blocking solution containing the secondary antibody (1:1000) were added for one hour at 37°C. After one wash step, samples were stored overnight in 500 µl PBS at 4°C. Next morning two washing steps were repeated, and the coverslides were mounted on glass slides using ProLong ® Gold mounting media (Invitrogen). After 24 hours drying at room temperature in darkness, imaging started and/or slides were stored at 4°C.

Blocking solution PBS with 10% FCS

2.2.3.8 HL-60 differentiation

HL-60 cells were counted, and for differentiation $1x10^6$ cells were added to 15 ml RPMI media (Biochrom AG) containing 10% FCS superior (PAA), and 190 µl DMSO. After 6 days

of incubation at 37°C,5% CO_2, cells were collected by centrifuging at 300*g* for five minutes, then resuspended in media and counted for further work.

2.2.3.9 General Phosphotyrosine assay

Cells were grown to a density of 70-90% depending of the cell type. Bacteria were grown 20-24 hours on agar plates, resuspended and their OD_{550} measured. Knowing that a suspension of *H. pylori* bacteria OD_{550} 0,1 represents approx. 3 x10^7 cfu/ml, cells were infected using a Multiplicity Of Infection (MOI) of 60 (approx. 60 bacteria per cell). In case of pre-treatment of cells with inhibitors, media was changed and inhibitors added 30 minutes before infection started. Infection was done for one, two or four hours at 37°C and 5% CO_2, and stopped by cold temperature. Supernatants were collected and stored at -20°C for later ELISA detection. Cells were collected in 1 ml ice cold fresh prepared PBS* using a cell scrapper and centrifuged at 500*g* and 4°C for 10 minutes. Pellets were resuspended in ice-cold RIPA buffer with freshly added protease inhibitors and DNAse I. To prepare the sample for detection of proteins, 2X SDS loading buffer was added and samples boiled in a water bath at 95°C for 10 minutes. Proteins in the samples were separated in a 6% or 8% gel and immunoblotting was done using a mouse polyclonal antibody against phosphorylated proteins PY99 (1:1000) or a mouse monoclonal anti-Phosphotyrosine 4G10 (1:10000). Detection followed using peroxidase conjugated anti-mouse antibodies in a dilution 1:10000.

PBS*	PBS (+Ca^{2+}, +Mg^{2+}), 1 µM Sodium- Orthovanadate, 1 µM PMSF, 1 µM Leupeptin, 1 µM Pepstatin, 10µg/ml DNAse I
RIPA buffer	150 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40, 0,25% (w/v) Sodium deoxycholate, 50 mM Tris HCl pH 7,4, 1 µM Sodium-Orthovanadate, 1 µM PMSF, 1 µM Leupeptin, 1 µM Pepstatin, 10 µg/ml DNAse I.

2.2.3.10 Assay for CagA phosphorylation inhibition by integrin antibodies

For this test, the general phosphorylation assay protocol was followed with some modifications. Cells were synchronized overnight, serum-free media removed and 1 ml CM added. After 30 minutes, 30 µg of antibody was added and left to bind for 30 minutes at 37°C in 5% CO_2 conditions. Bacteria were added for a MOI 60 for one or three hours. At the end of

the infections, cell were collected as described before. For the evaluation of IL-8 secretion, only supernatants of the three hour infections were analized.

2.2.3.11 Chemotaxis quantification through Boyden Chamber Assay

Differentiated HL-60 cells (dHL-60) were infected with an MOI 5 for one hour. Cells were collected and $1x10^6$ cells were added to the upper chamber of a cell culture insert with a membrane with 3 µm pores. After adding the chemoattractant in the lower chamber of the insert, incubation followed for one hour at 37°C with 5% CO_2 (see figure 3-1). Cells that migrated were found on the bottom well and were counted using a Neubauer Chamber. To avoid loss of cells, since they tend to bind to the bottom surface of the well, each well was mixed thoroughly and carefully before each counting event. Each well was counted two times.

2.2.3.12 Agarose Gel-based 2D migration Assay

A 1,5% agarose suspension in previously CO_2-saturated Buffered media was heated and poured onto a fibronectin-coated MatTek plate. After solidification, three wells were drilled in the agarose gel using capillary tubes. Plates were left for 30 minutes at 37°C with 5% CO_2 for CO_2 saturation. Differentiated HL-60 cells (dHL-60) were infected with an MOI 5 for one hour. Cells were collected and $5x10^5$ cells were added to a well in the agarose gel. In the other wells, chemoattractants were added. Imaging was started after 15 minutes for the lapse of one hour.

Buffered Media RPMI, 10% FCS, 25 mM Hepes pH 7,4.

2.2.3.13 Collagen Matrix 3D migration Assay

For the formation of the 3D collagen matrix, protocol originated from P. Friedl (Friedl et al., 1993) was followed. Differentiated HL-60 cells (dHL-60) were infected with an MOI 5 for one hour. Once the chamber walls were solid, the collagen matrix containing the cells was added and left to solidify for 30 minutes at 37°C. A second layer was added, solidified and the solution with chemoattractant was added. The chamber was immediately sealed and life imaging was done for the lapse of one hour with 20 seconds intervals at 37°C.

2.2.3.14 IL-8 production curves

AGS cells were splitted in 96 well plates, each with 200 µl media. Once they reached 80% confluency, their media was exchanged for media alone or for media containing the bacteria for a final MOI of 60.Supernatants were collected at the time points indicated and stored at -20°C until ELISA analysis.

2.2.3.15 siRNA transfection

Low passage AGS cells were splitted in a way that after 24 h they would have a maximum of 20% confluency in 6-well plates. After this time, a suspension of 50 nM dsRNA coding for the protein target was transfected using Lipofectamine RNAi-Max. As transfection control, siRNA-GFP oligonucleotides (Invitrogen) were used, as well as a standard RNA oligonucleotide with a sequence with similar GC content to the target sequence (dsRNA GC-Medium, Invitrogen). After two to three hours transfection, media was changed and transfection efficiency estimated by observation of fluorescent vesicles and/or nuclei in the cells. Only when the transfection efficiency was above 80%, cells were kept for the following experiments. After 48 hours, cells from a 6-well with same treatment were carefully detached using PBS/EDTA method, mixed and splitted 1:2 in 6 well plates. 60 hours after the transfection, cells were used for the experiments.

2.2.3.16 Binding Protocol for integrin activation status

In order to test the activation status of the integrins on the surface of the cells, the adhesion capacity to pre-coated plates was tested using the protocol of Mould et al (Mould et al., 1995) with some modifications. Cells grown in 75 cm^2 bottles to 70-80% confluency were detached with 3 ml trypsin-EDTA. After cells were in suspension, 10 ml media containing 10% FCS was added and then transferred to a tube for centrifugation at 300*g* for 5 minutes. The pellet was washed two times with 10 ml Hepes buffer. Cells were finally resuspended in the same buffer and counted. $1x10^5$ cells were used per treatment and exposed to Calcein AM for detection. In the case of infection, bacteria were given for a final MOI 30 or100. After exposure to treatments for 10 to 30 minutes at room temperature, cells were given to the pre-warmed BSA saturated wells and left binding at 37°C for 20 to 60 minutes in 5% CO_2. Unbound cells were washed three times and measurement done by fluorescence detection.

Hepes Buffer Hepes 25 mM pH 7,4 150 mM NaCl.

2.2.3.17 GST-fusion proteins binding to cells

Cells with 70% - 90% confluency in a 75 cm^2 bottle were detached using 2 ml trypsin EDTA, and collected in 10 ml RPMI serum-free media. To collect the cells, the suspension was centrifuged at 200*g* for 10 minutes, washed two times with 10 ml PBS (-Ca^{2+}, -Mg^{2+}), followed by a washing step with 5 ml of FACS buffer freshly prepared. Cells were finally resuspended in 1 ml FACS buffer. Viable cells were counted and $3x10^5$ cells added to a well from a 96-well plate with round bottom. Cells in the 96-well plates were centrifuged at 210*g* for five minutes, their supernatant was removed and 200 µl of a FACS solution with PMSF (1 mM, used as proteinase inhibitor to stabilize the fusion proteins during the incubation) containing 5-10 µg (per $1x10^6$ cells) of the fusion protein to be tested were added per well. Cells were incubated at 4°C for one hour and washed four times with FACS buffer with PMSF, 200 µl per well. Anti-GST primary antibody was added in a dilution of 1:1000 in FACS buffer with PMSF and incubated for 60 minutes at 4°C. To remove unbound antibody, two wash steps were repeated followed by addition of anti-mouse $Alexa_{488}$ secondary antibody (1:1000) in FACS buffer with PMSF and incubation in darkness for 45 minutes at room temperature. Cells were washed two times in FACS buffer with PMSF and collected in 500 µl of FACS buffer with PMSF to be analyzed by flow cytometry.

FACS buffer 27 mM Tris-HCl pH 7,4 , 137 mM NaCl, 2,4 mM KCl

2.2.3.18 Production of AGS exudates

AGS cells grown to a 70% - 90% confluency on 12-transwell membranes with 3 µm pores or on 75 cm^2 cell culture flask were washed two to three times with PBS (-Ca^{2+}, -Mg^{2+}) and left in 500 µl or 2 ml PBS (-Ca^{2+}, -Mg^{2+}) for 60 minutes at 37°C with 5% CO_2. The ideal time point to collect the exudate was determined by evaluating thechange of the PBS viscousity. The 2 ml exudates from the 75 cm^2 flasks were collected and filtrated though a 0,22 µm pore membrane. The filtrated solution was used to induce the expression of the Cag apparatus for EM studies.

2.2.4 Protein work

2.2.4.1 Protein concentration estimation

A BSA 100 mg/ml start solution was used as standard protein concentration. 10 µl of the protein solution and the standards in different concentrations (0,5 – 400 µg/ml), were added to 90 µl PBS and mixed. To this suspension, 1000 µl of Bradford solution was added, mixed and incubated 15 minutes at room temperature in darkness. The protein concentration was measured (OD_{595}) in combination with the standard curve, to determine the concentration of the samples.

Bradford Solution	0,01 % Coomassie Brilliant Blue G250, 5% Ethanol, 8,5% Phosphoric Acid

2.2.4.2 Separation of proteins and blotting

For the detection of proteins, separation of proteins based on their molecular weight was achieved using a SDS-Acrylamide gel.For immunodetection, the function of certain antibodies depends on the condition of the protein preparations. Therefore, most of the samples were prepared with 2X SDS buffer without β-mercaptoethanol. SDS gels (separation layer) containing concentrations of 6%, 8% and 12% acrylamide were prepared and left overnight at 4°C for full polymerization. Shortly before they were used, the 5% acrylamide upper gel (collection layer) was added. Samples were loaded and gels immersed in SDS running buffer, runned first for 10 minutes with 100V (~ 30 mA per gel), followed by 75-90 minutes at 130V. Proteins were transfered from SDS-acrylamide gels to a PVDF membrane using a semidry electric transfer chamber, with a 1,2 mA/cm^2 for 75 minutes, using anode I, anode II and cathode buffer as transfer buffers. For some gels zinc staining was done using the Zinc Staining kit from BioRad following the manufacturer's instructions. Once the transfer to the membrane was complete, membranes were dryed and stored at 4°C or -20°C until immunodetection.

SDS running buffer	250 mM Glycin, 0,1% SDS, 25 mM Tris HCl pH 8,3
Anode buffer I	0,3 M Tris pH 10,4; 10% Methanol
Anode buffer II	25 mM Tris pH 10,4; 10% Methanol

Cathode Buffer	25 mM Tris, 40 mM 6-Amino-n-Caproic acid (or glycin), 10% Methanol, final pH 9,4

2.2.4.3 Immunodetection by Western Blot

For the immunodetection, dryed membranes containing the separated proteins were immersed in 100% methanol for no less than 15 seconds. Once wet, the membranes were blocked with 5 ml blocking buffer for one hour. The buffer was then exchanged for 5 ml reaction buffer containing the primary antibody. Incubation of the antibody followed for one to two hours at room temperature. The excess and unbound antibody was washed five times with wash buffer every 10 minutes. 5 ml of reaction buffer containing conjugated secondary antibodies (alkaline phosphatase or peroxidase conjugated) were added to the membrane for 45 minutes at room temperature. Washing steps were repeated three times followed by detection of the secondary antibody. In many cases, re-evaluation of results by detection of other proteins was necessary. For this purpose, PVDF membranes were exposed to 5 ml of 50 mM NaOH for 1-2 hours in order to remove the antibodies. After the stripping of the membrane with the NaOH solution, the rest of NaOH was removed with distilled water and the immunoblot was started again with 5 ml of blocking buffer. A maximum of four stripping processes could be done without observing any damage on the proteins or membrane.

Blocking buffer	PBS, 5% Fat-free milk (powder).
Reaction buffer	0,9% NaCl, 10 mM Tris HCl pH 7,4; 0,75% Tween 20, 1% fat-free milk (powder)
Wash buffer	0,9% NaCl, 10 mM Tris HCl pH 7,4; 0,75% Tween 20

2.2.4.4 Fluorescent staining of proteins

Proteins which were to be stained using an AlexaFluor compound, were dialyzed previously in 2 liters PBS ($+Ca^{+2}$, $+Mg^{+2}$) at 4°C minimum for two hours in order to eliminate any traces of Tris contained in the storage buffer. After dialysis, staining was made following the manufacture's protocol (Invitrogen / Molecular probes). Conjugated proteins were store at -20°C until their use.

2.2.4.5 Production of AIIB2 antibody from hybridoma cells

Hybridoma cells were defrosted and cultivated for two days in 5 ml of DMEM media containing 10% FCS and glutamine (10 cm^2 cell culture bottles, 5% CO_2 at 37°C). All cells were then transferred to a 75 cm^2 cell culture bottles with a final volume of 15 ml. After 3 to 4 days, 5 ml from the cell culture were transferred to 175 cm^2 bottles with a final volume of 25 ml and left to grow until 80% of cells were non-adherent (Phenol red in media should have turned yellow). 20 ml of the cultures were then collected in tubes and centrifuged for 5 minutes at 300*g*. The supernatant was sterile filtrated through a 0,22 µm filter and stored until the detection of the antibody's production was done by Acrylamide-SDS separation and Coomassie Staining. For further production of antibody, 5 ml of cells resting in the bottle of the collected antibody were kept in culture by addition of, 20 to maximum 50 ml of media. The culture was incubated and the supernatant was harvested again when cells lost adherence (about 7 to 10 days later). Supernatants containing the antibody were store at -20°C for long term and for up to 6 months on 4°C.

2.2.4.6 Production and purification of GST fusion proteins

Strains containing the inducible plasmid were cultivated in 10 ml LB media at 37°C overnight.6 ml of the overnight culture was used to inoculate a 600 ml culture, which was incubated for approx. 3 hours at 37°C and constant agitation at 200 rpm.. Once the bacteria culture reached an OD_{550} of 0,5, induction was started with a final 1 µM concentration of IPTG. Further incubation was done at 27°C overnight under constant rotation for GST, GST-CagYa and GST-CagYb. For the production of GST-CagA, GST-CagYc and GST-CagI fusion proteins only 5 hours incubation could be done, before the proteins were degraded. Bacteria were collected by centrifugation at 6000 rpm for 15 minutes at 4°C. The pellet was resuspended in cold Buffer 1. Bacteria cells were lysed using an ultrasonicator with five times 30 pulses at 40% Output intensity, duty cycle 40% (Branson Sonifier 250) in ice-cold conditions. Bacteria fragments were separated from supernatant by centrifugation at 12000 rpm for 50 minutes at 4°C. After collecting the supernatant, equilibrated glutathione-sepharose beads were added for 20 minutes at room temperature or two hours at 4°C. Four washing steps followed. For this, Buffer 2 was used, with a volume corresponding to10 times the original beads volume. The elution buffer was added in the same volume as the original beads, 20 minutes incubated at room temperature, and beads separated from supernatant by

centrifugation for 5 minutes at 500*g* and 4°C. Eluted proteins were aliquoted and stored at -20°C until their use.

Buffer 1	PBS containing 10 mg/ml lysozyme, 2 mM EDTA, 1 mg/ml DNAse I, Complete™ protease inhibitor (Roche), 1 mM PMSF.
Buffer 2	PBS containing Complete™ protease inhibitor (Roche), 1 mM PMSF.
Elution Buffer	50 mM Tris HCl pH 8,2 – 8,7; Complete™, 0,1 M NaCl, 10 mM Glutatione.

2.2.4.7 Detection of proteins by Coomassie Staining

After separating the proteins in the samples using Acrylamide-SDS gel, the gels were immersed in 40 ml of Coomassie staining solution per gel (50 cm^2 gel) for 60 minutes. After this time, the solution was exchanged for 40 ml of Coomassie distaining solution. Exchange of the distaining solution occurred three times every 30 minutes until the bands were distinguishable from the background. Once the gels were ready, they were store in distilled water until they were dried for 30 minutes at 80°C in a Gel drying chamber.

Staining Solution	0,5% (w/v) Coomassie Brilliant Blue R250, 50%(v/v) Methanol, 7% (v/v) Acetic Acid
Distaining Solution	20% (v/v) Methanol, 7% (v/v) Acetic Acid

2.2.4.8 Coating of Fluorescent Beads

The sulphate-modified Red Fluorescent Beads (Sigma), were used following the manufacturer's directions. Beads were centrifuged at 500g for 15 minutes, washed using PBS and resuspended in PBS 1% BSA for saturation of the beads for one hour at room temperature in darkness. After that time, beads were washed two times to remove excess of BSA and resuspended in a buffer which pH was adjusted to the calculated Isoelectric Point (IP) of the protein in order to be bound (Repeat I pI 4,4; and Repeat II pI 8,8). For a 25 µl original bead suspension, 2 µg of protein was added into 500 µl of the IP coating buffer. Incubation was required for one hour at room temperature in constant rotation and darkness. Unbound proteins were eliminated by washing the beads with the IP buffer and resuspended in the original volume of beads taken in IP buffer with a 0,05% of Sodium Azide.

IP Buffer 8,8	150 mM Tris HCl pH 8,8
IP Buffer 4,4	50 mM Sodium acetate pH 4,4

2.2.4.9 Coating of Magnetic Beads

Carboxyl modified magnetic beads from Chemicell (Berlin) were coated using the manufacturer's protocol with some modifications. 10 mg particles of magnetic beads were washed three times with MES buffer and resuspended in 250 µl of MES buffer containing freshly resuspended 10 mg of CMC (1-cyclohexyl-3(2-morpholinoethyl) carbodiimide metho-p toluensulfonate) to activate the beads. Activation took place for 10 minutes at room temperature under constant agitation. Beads were washed two times in MES buffer and the proteins intended to bind were added in a concentration of 50 µg Tris-free protein per 10 mg magnetic particles. A two hour incubation at room temperature was followed by two washing steps in 500 µl PBS and a final addition of blocking buffer with 0,05% Sodium Azide for storage.

MES Buffer	0,1 M 2-(N-Morpholino)ethanesufonic acid pH 5,0
Tris Blocking buffer	0,1 M Tris HCl pH 7,4
BSA blocking buffer	0,1% BSA in PBS
Tris Storage buffer	25 mM Tris HCl pH 7,4, 0,05% Sodium Azide
BSA Storage buffer	0,1% BSA in PBS, 0,05% Sodium Azide

2.2.4.10 In vitro phosphorylation assay

In order to test if cells are able to phosphorylate the CagA, an *in vitro* phosphorylation of the CagA was was done using total cell lysate. The cells to be tested were grown in a 75 cm^2 bottle until they reached 90% confluency. They were collected and lysed in 50 µl of RIPA buffer. At the same time, a bacteria suspension of OD_{550} 0,1 was centrifuged, concentrated and resuspended in 50 µl NP-40 buffer. Both lysates were mixed in proportion 1:1. To the mixed lysates 100 µl of phosphorylation buffer was added for a final volume of 150 µl. The mixture was incubated at 37°C for 30 minutes and 5X SDS loading buffer was added. The samples were boiled and stored at -20°C until the protein separation and immunodetection of phosphorylated proteins could be done.

RIPA buffer	150 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40, 0,25% (w/v) Sodium deoxycholate, 50 mM Tris HCl pH 7,4, 1 µM Sodium-Orthovanadate, 1 µM PMSF, 1 µM Leupeptin, 1 µM Pepstatin, 10µg/ml DNAse I.
NP-40 Buffer	20 mM Tris HCl pH 7,5; 150 mM NaCl, 1mM Sodium Orthovanadate, 1% NP-40
Phosphorylation buffer	25 mM Tris HCl pH 7,2; 40 µM ATP, 6,25 mM $MnCl_2$, 31,25 mM $MgCl_2$, 62,5 µM Sodium Orthovanadate

2.2.4.11 Extraction of Cag apparatus proteins for integrin pull downs

Bacteria were grown overnight on GC plates and resuspended in PBS* including 10µg/ml DNAse I to an OD 2. This suspension was frozen overnight. Next day it was treated with a 10 mg/ml lysozyme solution and 4 mM EDTA for 20 minutes at room temperature. After the 20 minutes incubation, the suspension was ultrasonicated five times with 30 pulses at 40% output intensity, duty cycle 40% (Branson Sonifier 250) in ice. In 2 ml aliquots, samples were centrifugated at 45000 rpm for 75 minutes at 4°C, acquiring the Soluble I and Pellet I. Pellet I was resuspended thoroughly in 1 ml cold HSL buffer with protease inhibitors and once more ultrasonicated until an uniform suspension was observed. This suspension was centrifuged at 13000 rpm for 60 sec to eliminate non-resuspended particles and the supernatant (Soluble II) was taken for the pull downs, the pellet loading control for the immunoblot.

PBS*	PBS ($+Ca^{+2}$, $+Mg^{+2}$), 1 µM Sodium Orthovanadate, 1 mM PMSF, 1 µM Leupeptin, 1 µM Pepstatin.

2.2.4.12 Integrin β1 interacting proteins pull downs

In this experiments, the Soluble I and II (resulting from the protocol 2.2.4.11) were tested using the integrin $\beta_1\alpha_5$ or integrin $\beta_1\alpha_1$ coated magnetic beads. To each 2 ml initial Soluble I or II solutions prepared, 5 µl of the magnetic coated beads were added at 4°C for 45-60 minutes. To remove the unspecific binding proteins, three washes followed using 500 µl HSL 300/400, each time recovering the beads by use of a magnet. After the third washing step, all washing buffer was carefully removed. Magnetic beads were resuspended in 12 µl of Resuspension Buffer, and a equal volume of 2X SDS loading buffer was added. Samples were

boiled for 10 minutes at 95°C. For analysis of the interacting proteins, the samples were loaded onto 8% and 12% Acrylamide-SDS gels using the protocols described before.

HSL buffer	25mM Tris HCl pH 7,4; 0,05% Triton X-100, 4 mM $MgCl_2$, 3 mM $MnCl_2$, 150 mM NaCl, 1 mM PMSF.
HSL 300 buffer	25mM Tris HCl pH 7,4; 0,05% Triton X-100, 4 mM $MgCl_2$, 3 mM $MnCl_2$, 300 mM NaCl 1mM PMSF
HSL 400 buffer	25mM Tris HCl pH 7,4; 0,05% Triton X-100, 4 mM $MgCl_2$, 3 mM $MnCl_2$, 400 mM NaCl, 1 mM PMSF

2.2.4.13 Detection of IL-8 production

Using an Enzyme-linked Immunosorbent Assay (ELISA) as immunodetection system, concentrations of IL-8 produced by AGS cells during infection assays were detected. Immunoadsorbent 96-well plates (Nunc) were coated overnight with 30 µl of anti-IL-8 coating antibody (0,5 mg/ml) in 5 ml coating buffer, with 50 µl per well, at 4°C. Excess of antibody was washed three times using 200 µl wash buffer. Surfaces were blocked using 100 µl of blocking buffer for one hour at 37°C. Two washing steps were repeated. Standards and samples were added for a final volume of 100 µl per well and incubated for minimum three hours at 37°C. To remove unbound antigen, six wash steps were done. To detect the bound IL-8, 100 µl per well of a solution containing the monoclonal anti IL-8 biotinylated antibody (0,5 mg/ml) in wash buffer with 10% FCS were added. The biotynilated antibody (detection antibody) was incubated one hour at room temperature, washed six times and a streptavidin-peroxidase complex in ELISA buffer was added for one hour at room temperature. After six washes, TMB substrate for detection of peroxidase activity was added and the reaction was stopped after 30 minutes with 50 µl of the stop solution. The colorimetric reaction was measured using a wavelength of 450 nm with subtraction of a 570 nm wavelength in a Tecan Sunrise ELISA plate reader. Data analysis was done using Magellan 3 Software.

Coating buffer	50 mM Sodium Carbonate pH 9,6
Wash buffer	PBS ($+Ca^{2+}$, $+Mg^{2+}$), 0,05% Tween 20
Blocking buffer	PBS ($+Ca^{2+}$, $+Mg^{2+}$) 10% FCS
ELISA buffer	50 mM Tris HCl pH 7,6
Stop solution	1M H_2SO_4

3 Results

3.1 Cag T4SS interaction with Eukaryotic cells

Helicobacter pylori (H. pylori) presents a Type IV Secretion System (T4SS) denominated Cag that translocates the CagA protein into eukaryotic cells. The CagA protein contains EPIYA motifs at its C-terminal half that are phosphorylated at their tyrosine (Y) aminoacids upon entrance in the cytoplasmic compartment of the eukaryotic cell. The phosphorylation is catalyzed by members of the Src kinase family. Using this characteristic of CagA, the function of the T4SS is measured in assays were tyrosine phosphorylated proteins are detected by immunoblotting. In this way, the phosphorylated CagA is visualized and an effective CagA translocation confirmed. The following experiments detect the proper Cag T4SS operation through the detection of the phosphorylated CagA protein.

3.1.1 Effects of CagA on migration of dHL-60 cells

HL-60 cells are a pro-myelocytic leukemia cancer cell line, which has been extensively studied and used in laboratories since 1990. These cells are known to possess the capacity to differentiate in cells similar to the monocytic or the granulocytic cell lines depending on the stimuli used. In the presence of 1,3% DMSO for 5 to 6 days, HL-60 cells turn into cells showing characteristics typical for neutrophils, including the ability to migrate towards a chemoattractant.This ability is termed chemotaxis.

In the immune system, the ability of the cells to respond to a stimulus and, therefore, migrate towards a chemoattractant is essential for their proper operation. Here it was examined if *H. pylori* CagA or the Cag apparatus have an effect on migration of immune cells.As model cell line, the dHL-60 were used as neutrophil-like cells.

3.1.1.1 CagA inhibits the dHL-60 response to chemoattractants (Chemotaxis)

DMSO differentiated HL-60 (dHL-60) cells are defined as the closest cell culture model to study and to define the characteristics and requirements of neutrophils motility as a response to chemoattractants. In order to evaluate an effect of the Cag apparatus and its effector protein CagA on chemotaxis, infections were carried out using the *H. pylori* P12 strain and its mutant

strains, P12Δ*cag*A and P12ΔPAI. The effect of the Cag apparatus and/or CagA on the cells was measured by the capacity of the cells to migrate through a membrane towards a chemoattractant. As chemoattractant fMLP (formyl-Methionine-Leucine-Phenylalanine) was used. fMLP is a by-product of protein synthesis in prokaryotic cells. Its presence is detected by immune cells through the fMLP-receptor (Fechheimer and Zigmond, 1983). For the evaluation of possible effects on migration, cells were infected for one hour with an MOI (Multiplicity of Infection) of 5 (approx. 5 bacteria per eukaryotic cell) and placed on the top compartment of a Boyden Chamber containing a basal membrane with 3 µm pores. Their ability to move through the membrane for one hour as a response to the chemoattractant found on the lower chamber was measured by counting the number of cells found on the bottom compartment using a Neubauer chamber (Figure 3-1).

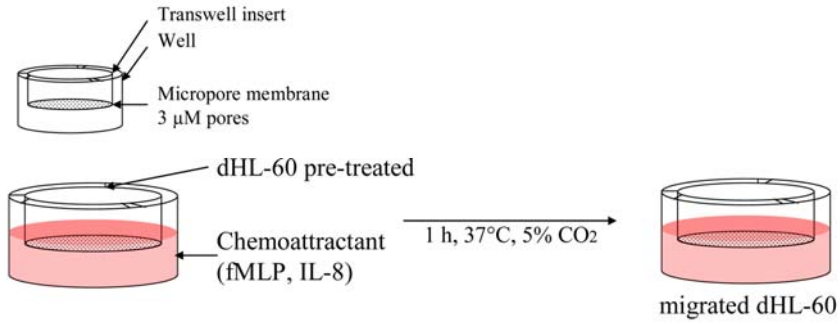


Figure 3-1 Boyden Chamber Migration assay

Schematic of Boyden Chamber used to calculate the migration capacity of differentiated HL-60 cells (dHL-60) toward a chemoattactant after 1-hour infection with *Helicobacter pylori* P12 wild strains and mutants

As seen in figure 3-2A, only for those infections where CagA protein was present, there was a reduction in the number of cells that were able to cross the membrane and migrate towards the lower chamber containing the chemoattractant. To ensure that the effect seen here is independent of the fMLP chemoattractant and its receptor, IL-8 was used next as chemoattractant. Here the same effect of the CagA is present (Figure 3-2B). Cytochalasin D, as Actin cytoskeleton inhibitor, served as control for migration. From the results of these experiments, it can be concluded that motility of dHL-60 cells is significantly reduced by the presence of the CagA protein, but no significant effect seems to be caused by other proteins from the apparatus.

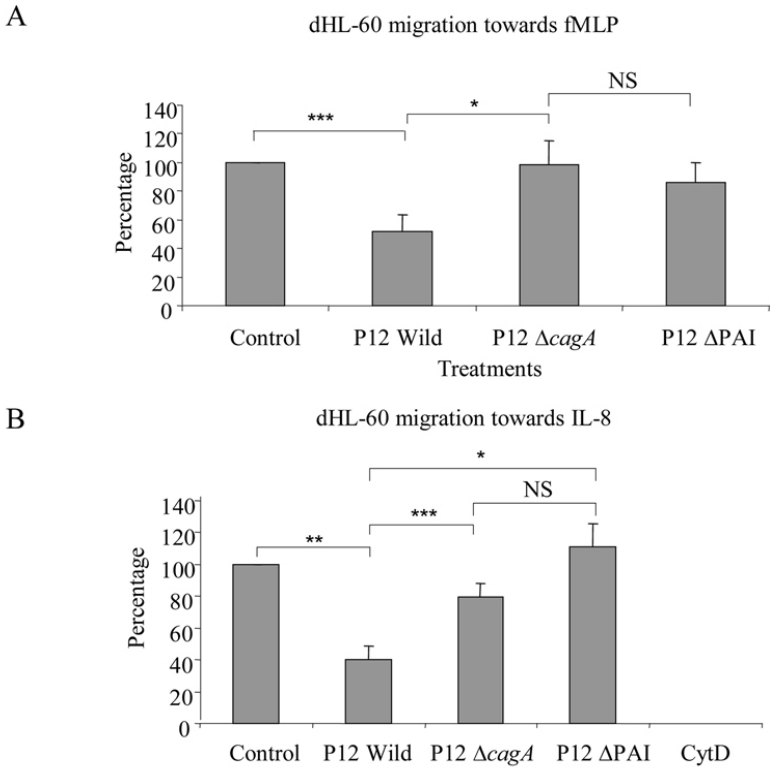


Figure 3-2 Effects of CagA and Cag apparatus on motility

Migration of dHL-60 cells after one hour infection with P12 wild type strains, P12Δ*cagA*, and P12 ΔPAI (MOI 5), towards (A) fMLP (formyl-Methionine-Leucine-Phenylalanine, 20 pM) and (B) IL-8 (8 µg/ml). CytD, Cytochalasin D. The graphics represent the percentage of migrated cells relative to uninfected dHL-60 cells (Control). Statistical analysis using t Test. * P<0.05, ** P<0,01, ***P< 0,005. NS, no significance

3.1.1.2 Migration deficiency caused by CagA

The CagA protein has an effect on migration of the dHL-60 cells, by reducing their capacity to move across a membrane. Since the motility of cells is the result of several synchronized processes tightly interacting with each other, more information can be gathered to assess the CagA's mechanism of action by evaluating themotility related changes of the cell caused by CagA.

To date the morphology of cells that takes place during motion in direction to a stimulus has been very well described (Gomez-Mouton et al., 2001; Heit et al., 2002; Meili and Firtel, 2003; Muller, 2003; Srinivasan et al., 2003; Van Haastert and Devreotes, 2004; Xu et al., 2003). Previously, a reduction of motility was quantified. A better definition of the effect caused by CagA on the cells can be evaluated by observing the characteristics of movement with help of live cell imaging. For this purpose, the movements of the cells in two different systems were compared. The first one, using an agarose test, simulates the 2D movement

described already for dHL-60 chemotactic motility. The second one, using a collagen matrix, evaluates their morphology and movement simulating a 3D system, similar to the conditions found in the animal tissue.

3.1.1.2.1 "2D" migration (Agarose Assay)

In the agarose assay, the cells must migrate between fibronectin covered glass and an agarose layer towards a chemoattractant, in this case fMLP. Cells do not show any affinity or binding capacity to agarose. Therefore, they are forced to move using only the fibronectin as anchoring point and by doing so, migrating on a two-dimensional system. After the glass surface of the MatTek plates was covered with human fibronectin, a 1,5% (w/v) solution of agarose was poured onto the plate and left to solidify. Once solid, by use of capillary tubes, small wells were perforated into the agarose gel. In one well fMLP was added and in the others the cells (uninfected or infected), which started moving once they sensed the chemoattractant. In order to allow cells to enter the space between glass and agarose, the imaging was started after 15 minutes after the addition in the wells and continued exposures with 20 s delays for 60 to 120 minutes in phase contrast were taken. In all experiments the *H. pylori* P12 wild type (w^+) strains and their Δ*cagA* mutant were used, a "barrier" of bacteria was seen between dHL-60 and chemoattractant, as if the bacteria were attracted to it and they moved faster towards it. This barrier presented itself as a problem since it restricted the movement of dHL-60 cells as a response to the chemoattractant gradient (data not shown).

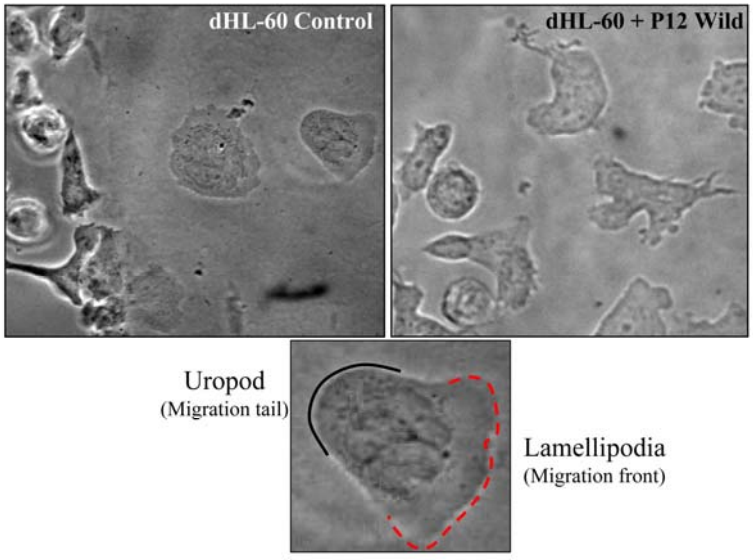


Figure 3-3 Effect of Cag on migration of cells in a 2D system

Phase contrast images of dHL-60 cells uninfected (control) and infected with P12 Wild type (MOI 5) migrating towards fMLP in an agarose system. Lower picture defines the morphology of typical leukocyte-like migrating cells.

However, in the transition between well and agarose, it was observed that dHL-60 cells were able to enter space between agarose and fibronectin coated glass independent of the treatment. As seen in figure 3-3, control cells and cells infected with P12 wild type produced a migration front, or lamellipodia, and a migration tail, or Uropod. Even though there seems to be some morphology differences, the Cag apparatus or CagA does not inhibit the polarization of the cells that is normally displayed by cells during migration.

3.1.1.2.2 3D migration (Collagen Matrix Assay)

Immune cells can find the place where they are needed by migration toward a stimulus. This migration includes getting out of the circulatory system through or between the endothelial cells of the veins, arteries and capillaries (called diapedesis). Once on the other side of the endothelial cells, migration occurs through the space between cells and tissues (interstitial space). In 1993, Friedl et al published a new design of an *in vitro* system in which the cells were suspended in a Collagen matrix, having the possibility to move through it in a three dimensional system. In these experiments, new phenotypes were described for cells during their migration through the 3D-Collagen matrix. Because of the limitations encountered using the 2D agarose system, the collagen matrix offered a possibility to observe the motility of the cells and the effects of CagA on it.

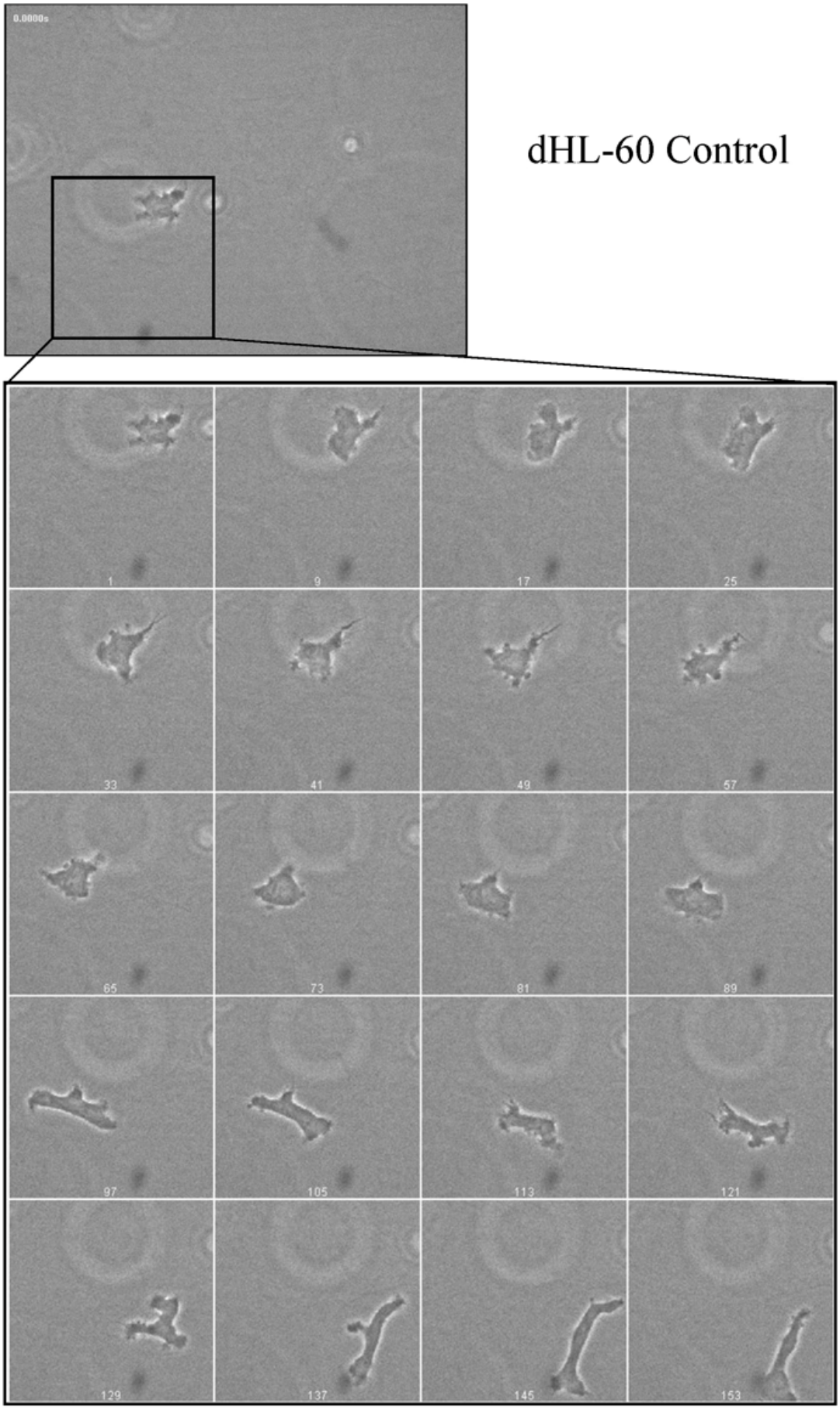
For the collagen matrix assay, dHL-60 cells were collected and infected as described for the experiments in 2D Agarose system. From this suspension, cells were added to the Collagen matrix mix and left to solidify followed by an addition of a chemoattractant, sealing of the chamber and taking pictures in the microscope (Microscope DM IRB, Leica), at 37°C for a minimum of one hour with 20 seconds intervals. Because the matrix is between 2-3 mm thick, only planes were imaged where cells showed any migration characteristic (Lamellipodia and uropod). Control cells (Figure 3-4A) showed a defined movement with a migration front and uropod retraction. Contrary to these results, *H. pylori* P12 wild type infected cells were not able to form neither front nor uropod, maintaining a spherical form (Figure 3-4B). In the case of P12 Δ*cagA* infections, some of the cells were not able to form an active migration front or lamellipodia, but the majority had a similar morphology seen in the control cells. However, their movements seemed slow and erratic (Figure 3-4C). P12 Δ*cagPAI* infected cells showed a morphology and movement similar to uninfected cells (Figure 3-4D).

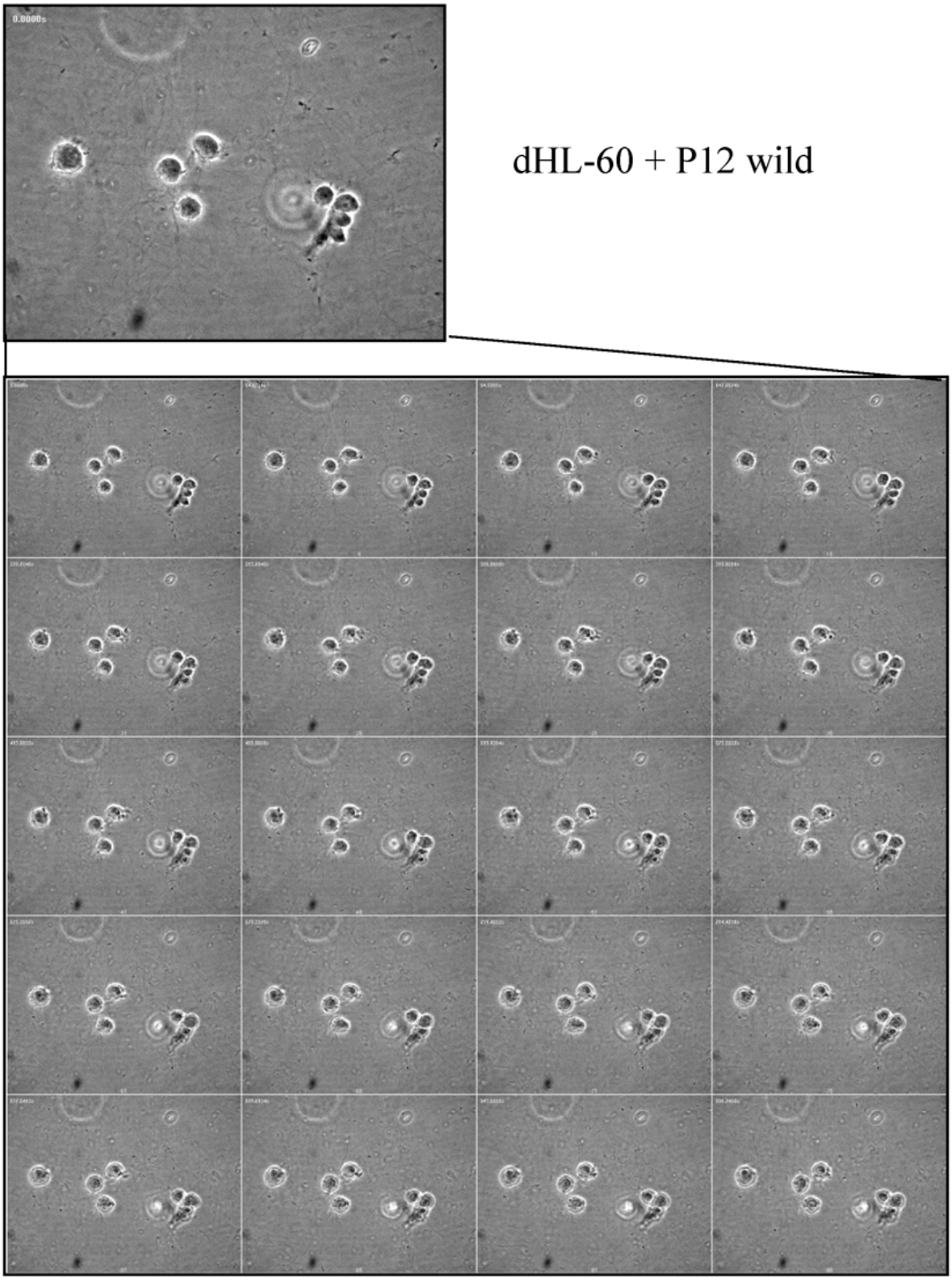
These results confirm the inhibitory effect on migration when cells are infected with P12 Wild type. The presence of CagA in 3D collagen matrix conditions shows that inhibition does not act on the coordination between processes of formation of lamellipodia and retraction of

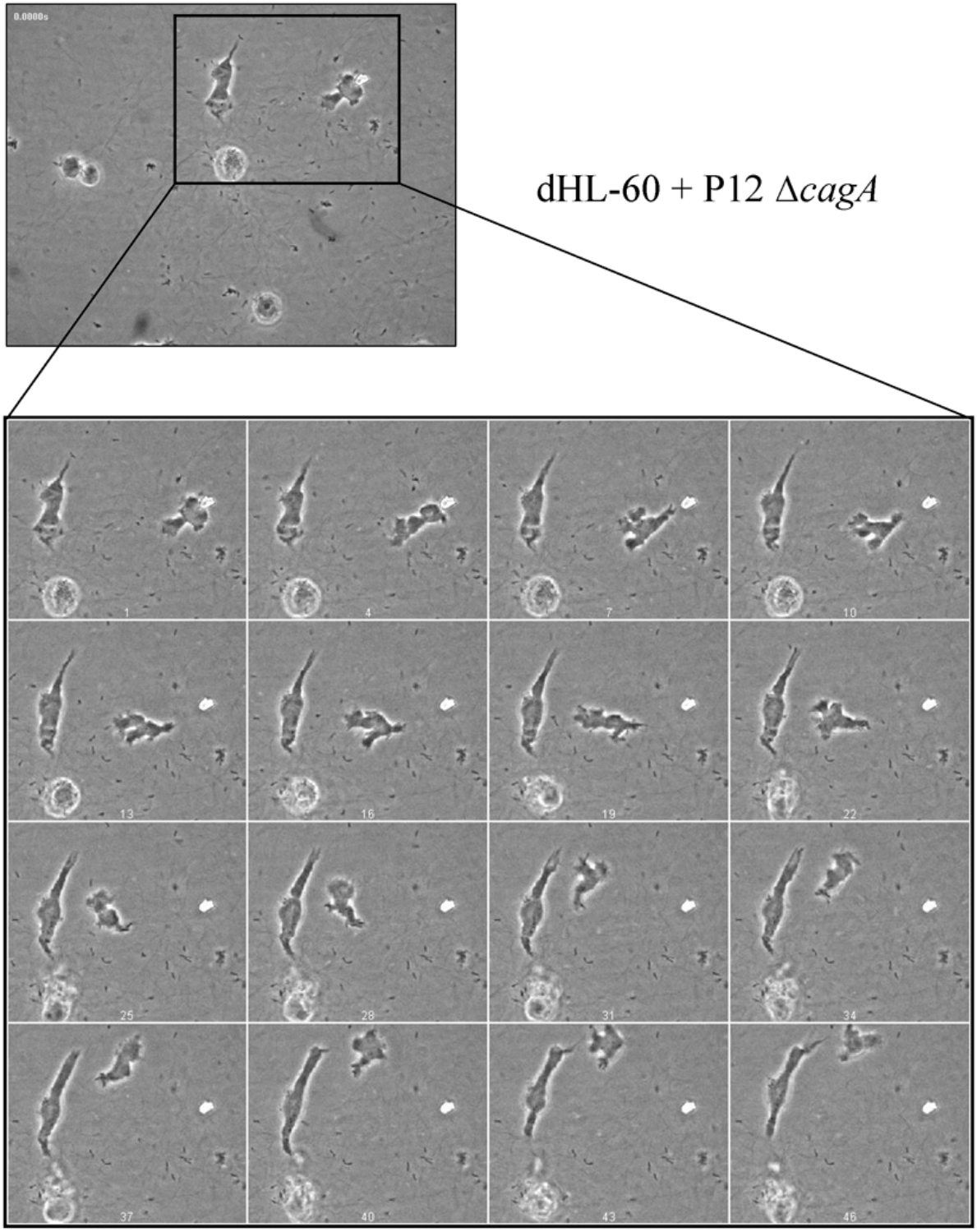
uropod, it shows that a previous event is blocked, since cells are not able to even adhere to the collagen fibers. Using CagA (-) strains only slowed down the migration of the cells in the collagen matrix but did not inhibit neither the formation of lamellipodia nor the uropod formation and retraction.

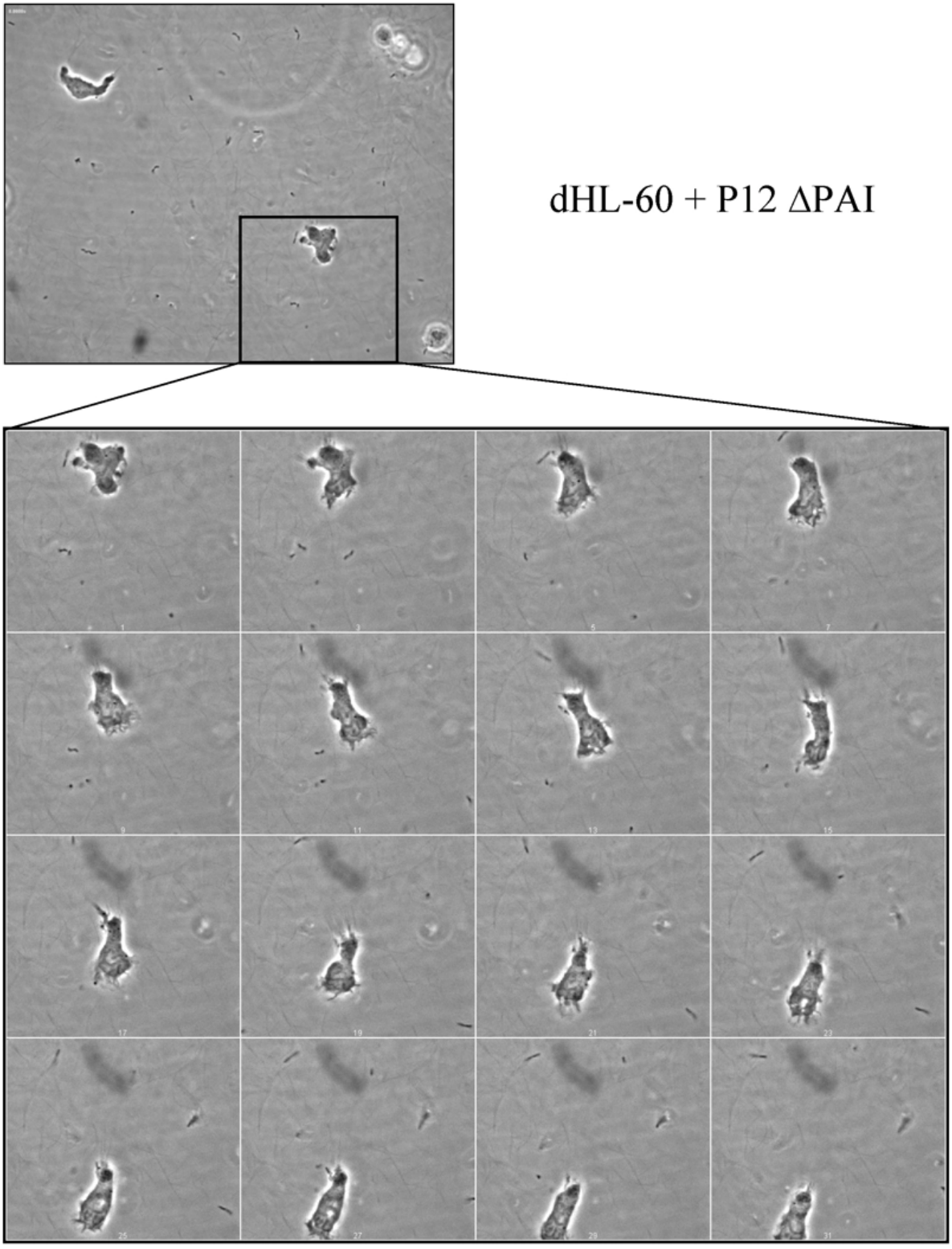
Figure 3-4 Migration of dHL-60 in a collagen matrix (next pages)

Images from Live Imaging experiments recording the movement of dHL-60 cells (A) uninfected (Control) or (B) infected with P12 wild type strains, or mutants lacking (C) *cagA* (P12Δ*cagA*) or (D) the entire CagPAI (P12 ΔPAI). Time lapse between each picture is 2 minute 40 seconds (160 seconds).


0.0000s
dHL-60 Control
1
9
17
25
33
41
49
57
65
73
81
89
97
105
113
121
129
137
145
153


dHL-60 + P12 wild


0.0000s
dHL-60 + P12 ΔcagA
1
4
7
10
13
16
19
22
25
28
31
34
37
40
43
46


dHL-60 + P12 ΔPAI

3.1.2 Integrin β1 is essential for CagA translocation

In the previous motility studies, differences were observed between the parental cell line HL-60 and the DMSO differentiated HL-60 (dHL-60) in their capacity to phosphorylate CagA (CagA-P) (data not shown). To confirm that this effect was not strain specific, two other *H. pylori* wild type strains, which contain a functional Cag apparatus, P217 and P145, were tested as well. In these experiments, the same results were observed: massive reduction of the CagA-P when bacteria infected dHL-60 cells in comparison to the HL-60 cells (Figure 3-5A). By an *in vitro* phosphorylation assay, in which bacteria and cell lysates are mixed, it could be confirmed that both cell lines contained the kinases necessary to phosphorylate CagA (data not shown). This suggested that the inability of dHL-60 cells for CagA phosphorylation might be a result of the interaction of the Cag T4SS with the host cell membrane.

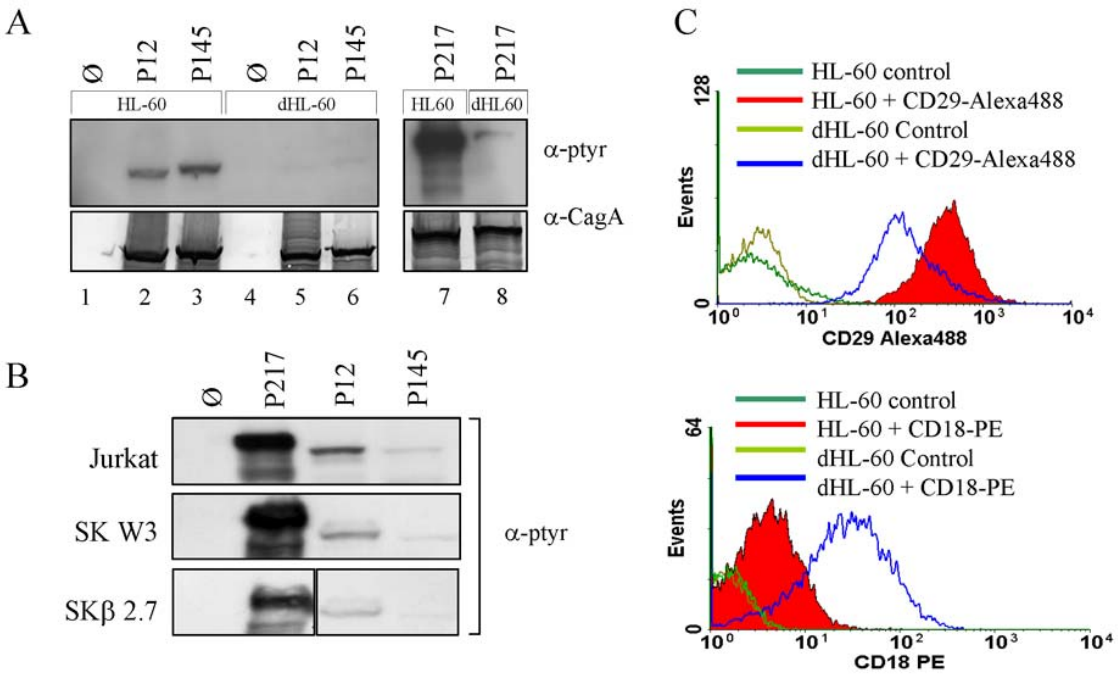


Figure 3-5 HL-60 and dHL-60 differences and integrin relations to CagA translocation

(A) Differences in CagA phosphorylation (CagA-P) in HL-60 and DMSO differentiated HL-60 (dHL-60) cells. Western blot detecting phosphorylated CagA (CagA-P) using antibody anti-phosphotyrosine (PY99) and anti-CagA antibody (AK257) after a 4-hour infection with MOI 60. (B) Effect of integrin β2 on CagA phosphorylation. Western blot detection of CagA-P in cells with integrin β2 (SK W3) and their integrin β2 knockout form (SKβ 2.7) after a 4-hour infection with MOI 60 (strains P217, P12 and P145). Jurkat cells were included as control for lymphocyte lineage. (C) Expression levels of integrin β1 and integrin β2 on HL-60 and dHL-60 (dHL-60). Flow cytometry from HL-60 and dHL-60 cells labeled with anti-integrin β2 (αCD18-PE) and anti-integrin β1 (αCD29 FITC). For each cell line, a control background was evaluated.

Taking advantage of the great amount of genomic and proteomic data available for these cells, a specific search for differences in membrane proteins that appear after the differentiation was performed (Hauert et al., 2002; Itoh et al., 1998; Okubo et al., 1995; Yanagida et al., 2007). Expression differences were found on two members of the integrin family, integrin β1 (CD29, ITGB1) and integrin β2 (CD18, ITGB2). The first step was to confirm the expression levels of both integrin proteins on the different cells using immunofluorescence and flow cytometry. By flow cytometry analysis it was confirmed that integrin β2 is absent in HL-60, but present in dHL-60. This correlates with already published data (Collins et al., 1979; Itoh et al., 1998). In the case of integrin β1, a higher level was observed on the surface of the non-differentiated cells compared to the differentiated ones (Figure 3-5C).

The possibility that the presence of the integrin β2 subunit could have a negative effect on the translocation and phosphorylation of CagA was tested using the T-lymphocytic cell line SKW3 and their corresponding integrin β2 knockout part, SKβ 2.7. Jurkat cells were used here as positive control cell line of lymphoid origin. After infection of these cells, their tyrosine-phosphorylated proteins were detected using immunoblots. As seen in Figure 3-5B, there is no difference in CagA-P levels in knockout cells compared with the ones expressing integrin β2. With this result, any involvement or negative effect of integrin β2 in CagA translocation could be excluded.

In HL-60 cells higher levels of CagA-P correlate with higher amounts of integrin β1 present on the surface of these cells. To confirm the relevance of integrin β1, a different approach was necessary. For this, integrin β1 knockout (KO) cell lines GD25 and GE11 were tested, using as control the corresponding integrin β1 complemented cells, GD25β (mouse integrin β1) and GE11β (human integrin β1), respectively. After infection of the cells under standard conditions (MOI 60, 4 hours, 37°C, 5% CO_2) using the P217 strain, CagA-P_{P217} was detected using the same procedure as described before. The immunodetection of tyrosine-phosphorylated proteins shows that CagA-P from strain P217 is present only in infections of integrin β1 complemented cells but not on the knockout counterparts (Figure 3-6A).

As explained before, phosphorylation of translocated CagA is catalyzed by Src kinase family members. Since integrins are important for membrane signalling processes in the cell, the possibility existed that knockout cells had a deficit in the recruitment of Src kinases to the translocation site producing a false negative result. To rule this out, a P12 strain expressing a CagA protein containing a N-terminal GSK tag was used for infection of the integrin β1

knockout cell lines (GD25 and GE11) and their integrin complemented versions (GD25β and GE11β). The function of a GSK tag is to confirm the translocation of proteins into the cytoplasm by using a threonine/serine phosphorylation of the tag as read out (Pattis et al., 2007). This method gives an alternative phosphorylation site on the protein that is Src-kinase independent. Using the GSK-tagged CagA (GSK-CagA), infections were done and the phosphorylated tag was detected using an antibody against phospho-GSK in immunoblots. It was confirmed that GSK-CagA was GSK-phosphorylated only in infections where the cells expressed integrin β1. It was verified that the data previously observed with the normal CagA tyrosine phosphorylation (Figure 3-6B) were not a consequence of a defective recruitment or function of Src kinases related to the integrin β1 knockout condition of the cells. Any possible false negative results were discarded.

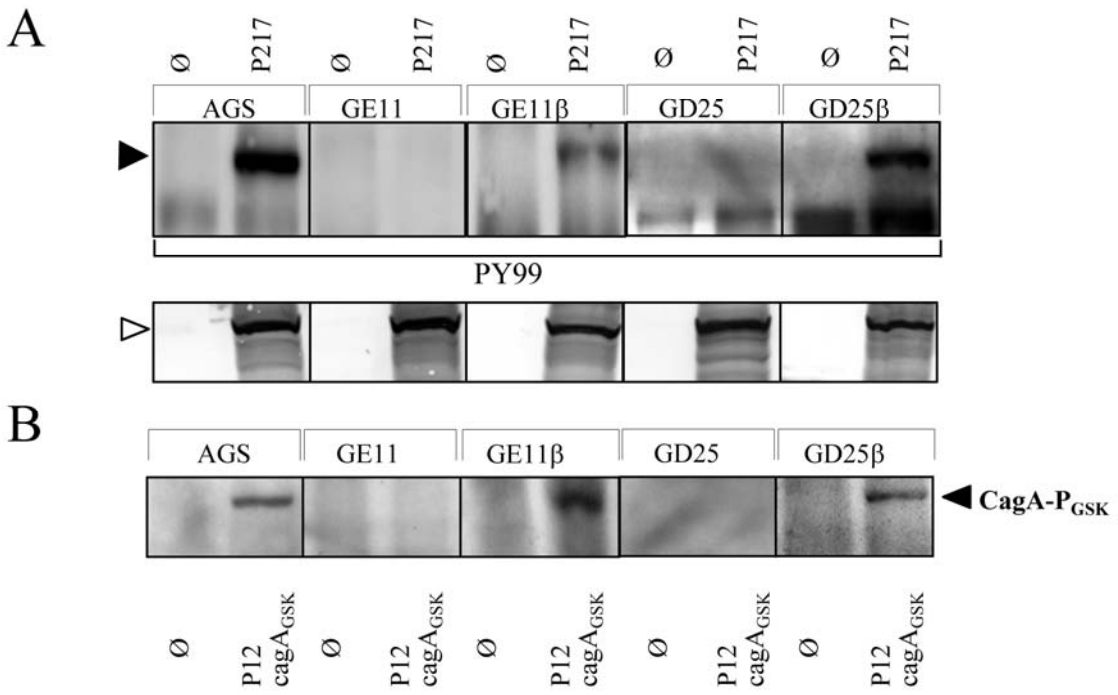


Figure 3-6 Integrin β1 dependent CagA phosphorylation in different cell lines

(A) CagA translocation and tyrosine phosphorylation in integrin β1 knockout cells (GE11 and GD25) and their integrin β1 complemented forms (GE11β and GD25β). Western blots detecting CagA-P from strain P217 after 4 hours infection with a MOI 60. Full arrowhead shows phosphorylated form of CagA detected using anti-phosphotyrosine antibody (PY99). Empty arrowhead shows bands corresponding to CagA detected using anti-CagA antibody (AK257). (B) Phosphorylation of GSK-CagA protein in integrin knockout cells and their integrin β1 complemented forms. Detection of phospho-GSK tag after 4-hour infections of AGS, GE11, GE11β, GD25 and GD25β cells with a P12 strain expressing a CagA-GSK protein at MOI 60.

In all these different experiments, the relevance of the integrin β1 for the CagA translocation was recognized, concluding that integrin β1 plays an essential role for the translocation of CagA into the cells. Additionally, they show that this role appears to be independent of mouse or human origin of the integrin β1.

3.1.3 CagA translocation efficiency correlates with the presence of integrin β1 on the surface

3.1.3.1 Integrin β1 is expressed on the surface of AGS cells in a cyclic manner

With the intent to confirm the connection of integrin β1 expression on the membrane of cells and the capacity of the bacteria to translocate CagA, the amount of integrin β1 expressed on the surface of synchronized adherent AGS cells was measured. The purpose of synchronization is to arrest all cells in G_0 of the cell cycle, start the experiments at a known time point of the cells' metabolism and analyze them as a uniform population. For synchronization, cells grown to 70% confluency were left in serum-free media overnight. To these cells, CO_2 saturated warm media containing serum (Complete Media, CM) was added. After the addition of CM media, cells were fixed at the time points indicated (0, 2, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 minutes) using the "*in flagrante*" method, in which cells are fixed instantly by addition of a pre-warmed concentrated form of paraformaldehyde. Fixation was followed by detection with primary antibodies against integrin β1 (Clone AIIB2). The labeling of the primary antibody was achieved by use of an anti-rat IgG $Alexa_{488}$ secondary antibody for flow cytometry analysis. By labeling directly on the original growth surface of the well, only the exposed integrin β1 on the surface of the cells was detected. Before cells were analyzed by flow cytometry, they were carefully scrapped and collected in 1 ml PBS. The acquired data was evaluated using WinMDI software. The mean peak of fluorescence of the cell population for each time point was determined. In figure 3-7A an example curve is illustrated. In this figure includes as well an overview of the protocol followed. As depicted in the curve, approximately two minutes after addition of serum containing media, integrin β1 levels on the membrane were slightly reduced. After that, the integrin β1 levels on the surface were relatively stable until 40 minutes after the addition of the serum-media. At this time point, an exponential increase of integrin β1 on the surface takes place, reaching a peak at

approximately 60 minutes. Between time points 60 and 80 minutes, these levels are again reduced until a baseline point similar (or sometimes lower) as the one found on cells after synchronization (time point 0). This process repeats itself with a periodicity of about 60 minutes for cells in low passage, and it shortens as the cells accelerate their reproduction time at high passage number (data not shown).

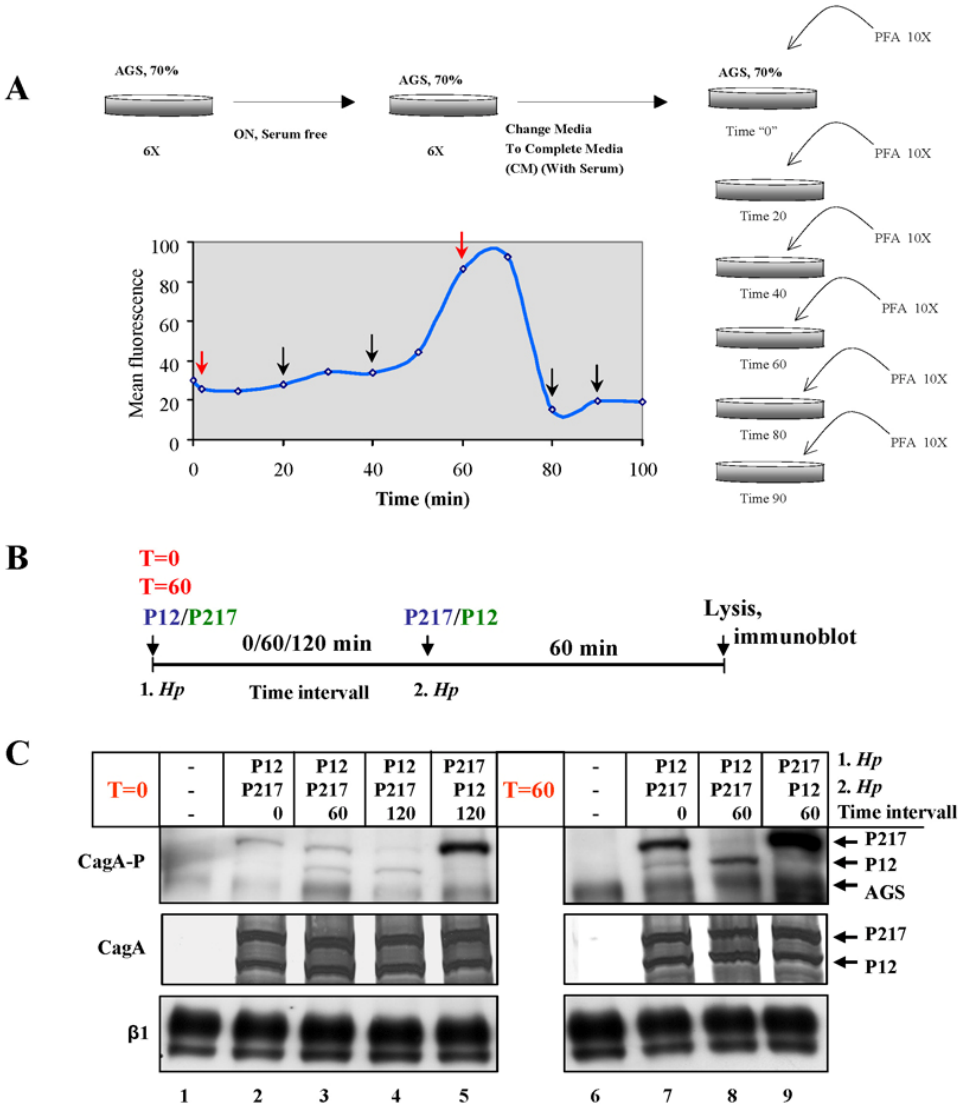


Figure 3-7 Dynamics of integrin β1 surface location and effect on *H. pylori* CagA translocation

(A) Integrin β1 cycle on AGS cells. Scheme of protocol used to determine integrin β1 presence on AGS cells' surface in relation with time. Curve represents the variations on integrin β1 surface presence after synchronization and addition of serum containing media. Antibody clone AIIB2 was used for the detection of whole integrin β1 population. Analysis using flow cytometry detection and calculated the mean fluorescence for each time point. Curve here is representative of three independent experiments. (B) Time points of infection of AGS cells with first and/or second strain. All infections took place for one hour after addition of the second strain. (C) Western blots detecting levels of CagA phosphorylation at time point 0 and 60 after serum addition. Strains used were P12 and P217 with an MOI 60. Phosphorylated CagA (CagA-P) detected using anti-phosphotyrosine antibody PY99, CagA levels using anti-CagA (CagA) antibody AK257 and to confirm comparable amount of cellular proteins an anti-integrin β1 (β1) antibody (Clone LM534) was used.

By establishing that the integrin β1 expression on the surface of the AGS cells is cyclic and can be coordinated for a maximum expression on the surface of the cells, it makes possible to determine the ideal time point for an evaluation of the relation of integrin β1 on CagA phosphorylation.

3.1.3.2 CagA translocation and phosphorylation in relation to integrin β1 membrane expression levels

Knowing the time points where to expect the maximum or minimum amount of integrin β1 on the surface of AGS cells, infections were done using two wild type strains, P12 and P217. Since both contain CagA proteins of different size, co-infections were made to evaluate a strain independent effect. Figures 3-7B explains the infection protocol. The results of the infection seen on figure 3-7C give an idea about the connection of the integrin β1 presence on the surface and the CagA-P levelsAt time point zero cells were infected for one hour with the two strains. In this time lapse (0-60 minutes), the cells express their minimum amount of integrin β1 on the surface for the time of infection. As seen in the immunoblots detecting phosphorylated CagA, the amount of CagA-P is lower as compared to infections of cells expressing the highest amounts of integrin β1 on the surface at the initial time point of infection (time 60). The effect is independent of the bacterial strains and the co-infection conditions (Figure 3-6 C, lanes 2 vs. 6, and 3, 4 vs. 8, 9). For infections where the initial time point was 0, it is necessary that approx. 120 minutes passed to obtain similar CagA-P levels as obtained after 60 minutes in infections were the initial time point was 60 (Figure 3-6 C lanes 4, 5 vs. lanes 8, 9). This data supports a direct connection between the amount of integrin β1 on the cellular surface of AGS and the efficiency of CagA translocation/phosphorylation.

3.1.4 Relevance of integrin β1 extracellular activation and its cytoplasmic domain for CagA translocation

3.1.4.1 Only the extracellular part of integrin β1 is necessary for CagA translocation

The principal characteristic of integrins is their inside-out signaling, achieved by the connection between their extracellular domain and cytoplasmic domain (Arnaout et al., 2005).

In order to elucidate if this property of the integrins is relevant for the CagA translocation, cells expressing full human integrin β1 (Isoform A) as well as its truncated forms in its cytoplasmic tail were tested. CHO-K1 cells, which are Chinese Hamster Ovary cells, were shown to be negative for CagA translocation. Therefore, expression from the different forms of human integrin β1 in these cells was used to evaluate their relevance in CagA translocation. As described in figure 3-8A, CHO-β1A contained the full version of human integrin β1 (Isoform A). CHO β1com expresses the integrin β1 form containing only the domain denominated "integrin common region" (com) found in the cytoplasmic part. CHO-β1TR does not present any part of the cytoplasmic tail. It contains only the transmembrane and extracellular domain (Retta et al., 1998). For the infections, P12 and P217 wild type strains were used under standard infection conditions.

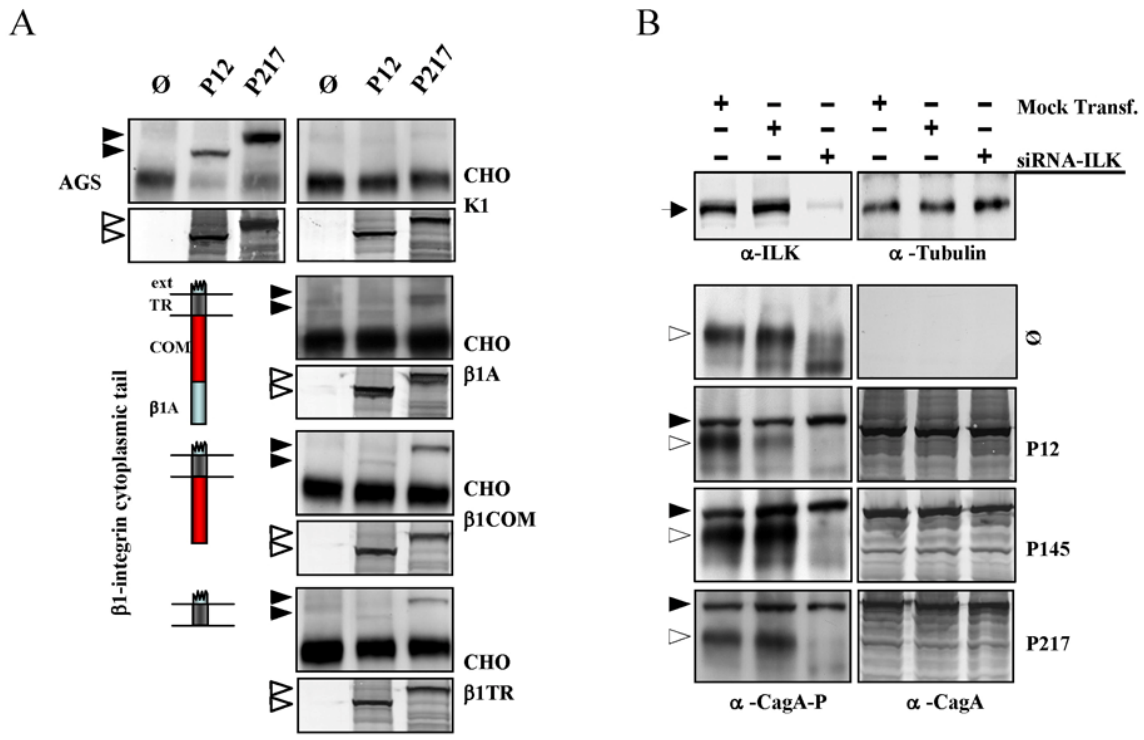


Figure 3-8 Effect of integrin β1 cytoplasmic tail and integrin β1 signaling on CagA translocation

(A) Immunodetection of phosphorylated CagA after infection with strains P12 and P217 of AGS cells (Positive Control), CHO-K1 (Negative control) and cells expressing the full integrin β1A protein, and cells expressing the cytoplasmic truncated forms β1com and integrin β1TR. The variations on cytoplasmic domain of the integrin β1 proteins are illustrated. Full arrowheads shows phosphorylated CagA form detected using Anti-phosphotyrosine (PY99) antibody. Empty arrowhead shows bands corresponding to CagA detected using antibody AK257. (B) ILK role in CagA translocation and phosphorylation. Immunoblots detecting levels of ILK on normal AGS cells and AGS cells transfected with siRNA against ILK mRNA. Strains used for a 3-hour infection were P12, P217 and P145. Full arrowheads shows phosphorylated CagA and empty arrowheads shows phosphorylated cellular proteins. Tubulin detection was used as marker to compare the total amount of cellular proteins.

After infection of the different CHO cell lines, the immunodetection of their phosphorylated CagA proteins revealed that both strains were able to translocate CagA in CHO expressing the complete version of human integrin β1A (CHO-β1A). Surprisingly the same result was obtained in cells expressing the truncated forms of integrin β1: β1A COM and β1 TR, both of them missing parts of their cytoplasmic domain but presenting the extracellular part of the integrin β1 on the surface of the cells (see Figure 3-8A). These results indicate that the cytoplasmic domain of the human integrin β1 is not essential for CagA translocation. The extracellular and transmembrane domains are enough for the Cag apparatus to translocate CagA into eukaryotic cells.

3.1.4.2 Integrin β1 cytoplasmic signaling is not required for CagA phosphorylation

After having shown that the cytoplasmic part of the integrin β1 does not play a role in CagA translocation, it can be expected that integrin β1 signaling will show no relevance on the efficiency of CagA translocation. Part of the integrin signaling occurs through changes transmitted from the extracellular part to the intracellular part causing modifications that induce the interaction of the cytoplasmic tail of integrin β1 with other cytoplasmic proteins. Some of the proteins known to be part of this intracellular signaling are the Focal Adhesion Kinase (FAK) and the Integrin-linked Kinase (ILK) (Hsia et al., 2003; Legate et al., 2006). FAK is a protein tyrosine kinase involved in the formation of focal adhesions that link the integrin β1 with the cytoskeleton (Delon and Brown, 2007; Liu et al., 2000). The serine/threonine kinase ILK interacts directly with the cytoplasmic region of the integrin β1 regulating integrin β1-mediated signal transduction (Wang et al., 2006). An indirect approach was taken to confirm the previous results. In our first approach, AGS cells were transiently transfected with a construct for expression of the dominant negative (DN) form, green fluorescent fusion protein (GFP), of FAK (FRNK-GFP). The expression of the protein was confirmed after 24 hours by fluorescence microscopy. However, transfected cells were not able to survive after 36 hours of transfection, which is the time recommended for an evaluation of the FAK. This cytotoxic effect left not enough material for a reliable phosphorylation assay. Since ILK is involved with the integrin β1 as well, a reduction of ILK expression levels was achieved using siRNA in AGS cells. With an approx. 99% transfection efficiency of the siRNA sequence, cells were grown 60 hours. Their ILK levels were reduced

between 71-76% as measured on immunoblots using a densitometer software analysis. Lower levels of ILK expression were reached around 72 hours after the transfection, were accompanied with high lethality as well. Therefore, infections were done after 60 hours of transfection for four hours with *H. pylori* strain P12. As seen in figure 3-8B, even though the levels of ILK were reduced almost to 20% of its original levels, CagA-P levels were not affected by this reduction. In agreement with the results previously discussed, CagA translocation needs only the extracellular part of the integrin β1, neither its cytoplasmic domain, nor its pathway linked to the ILK activity.

3.1.4.3 The activation status of the integrin β1 alters the CagA translocation efficiency

3.1.4.3.1 Bivalent cations effect

The activation status of integrins can be altered by extracellular changes on the concentration of divalent cations such as calcium or magnesium. It has been published that high concentrations of calcium work as antagonist of the adhesion properties, and magnesium as agonist (Mould et al., 1995). Because the effect caused by Mg^{2+} can be drastically magnified by use of Mn^{2+} at very low concentrations, Mn^{2+} was preferred. To test the effects of the activation status in the CagA translocation, 80% confluent cells were washed three times with PBS (-Ca^{+2}, -Mg^{+2}) and media containing serum and glutamine (Complete Media, CM) was added. 10 mM $CaCl_2$ or 1 mM $MnCl_2$ concentrations were added to the cells for 30 minutes pre-incubation. Infection with P12 wild strains followed for one, three and four hours. After detecting the CagA-P levels through immunoblotting of the infected cells lysates, CagA phosphorylation did not change with Mn^{2+} after four-hour infections (data not shown). However, it increased the CagA translocation after one-hour infections (Figure 3-9B). Calcium behaved differently. It decreased the levels of CagA-P after four hours (data not shown).

To rule out a direct effect of calcium on the bacteria, suspensions in warm RPMI complete media saturated with CO_2 of P12 and P12 mutant lacking the *cagE* gene of the Cag apparatus (P12 Δ*cagE*) were exposed to 5 mM and 10 mM concentrations of $CaCl_2$ for four hours in 10% CO_2. After collecting the bacteria by centrifugation, they were lysed and their proteins separated in an SDS-Acrylamide gel and blotted for immunoanalysis by detection of the CagA protein using anti-CagA (AK257) antibody. As seen in figure 3-9A, an inverse effect

on CagA concentrations in the lysate was detected in relation to the concentration of calcium.Therefore, an inhibitory effect of calcium on the CagA concentrations in the bacteria cell took place. Because of these results, the initial experiments to evaluate the calcium effect on phosphorylation of CagA were repeated with an one-hour infection only. In this case, no difference was seen in the levels of CagA-P (data not shown). By using the extracellular bi- and trivalent metal ions chelator EDTA at final concentrations of 2 mM, no effect was observed on the CagA-P levels. However in experiments where cells were pre-incubated with BAPTA, a cell permeable calcium-specific chelator, and infected for one or three hours with three wild type strains (P12, P217 and P145), a strong reduction of CagA-P levels was observed in a strain specific manner (Figure 3-9C and Table 3-1). Thus, activation of integrins by $MnCl_2$ at 1 mM concentration increases the CagA translocation and phosphorylation after one-hour infection. In the case of calcium, it seems to alter the efficiency of CagA translocation only when it is sequestered from the intracellular compartments. However no effects on CagA translocation were visible by low levels in the extracellular milieu.

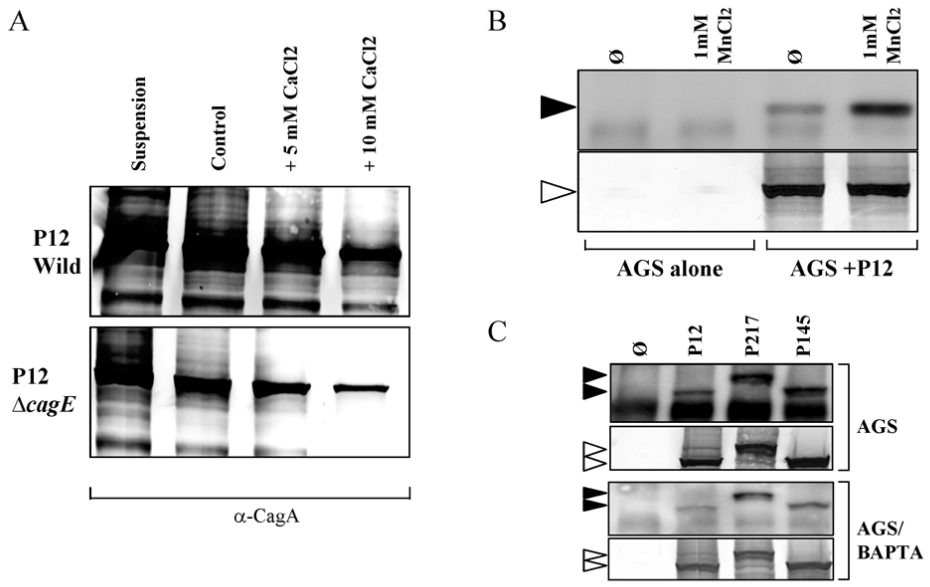


Figure 3-9 Effect of bivalent cations on CagA levels and CagA phosphorylation

(A) P12 wild type and P12Δ*cagE* OD 0,1 in RPMI media incubated 4 hours with different concentrations of calcium chloride. CagA levels detected using antibody AK257. (B) Effect of $MnCl_2$ in CagA phosphorylation. AGS cells with 1 mM $MnCl_2$ were infected for one hour with strain P12. Western blots indicate CagA-P levels with or without $MnCl_2$. Full arrowheads show phosphorylated form of CagA detected using anti-phosphotyrosine antibody PY99. Empty arrowhead show bands corresponding to CagA detected using anti-CagA antibody AK257. (C) Effects of BAPTA on CagA phosphorylation. AGS cells pre-incubated 30 minutes in a 60µM solution with BAPTA and infected with P12, P217 and P145. Full arrowheads show phosphorylated form of CagA detected using anti-phosphotyrosine antibody PY99. Empty arrowheads show bands corresponding to CagA detected using anti-CagA antibody AK257. Infections with MOI 60.

Table 3-1 Ligands or inhibitors of cell's cytoskeleton or integrin signaling and their effect on CagA translocation.

Treatment	*Concentration*	*Target*	*CagA Translocation*	*IL-8*
None	-	-	+++	++++
Integrin activating and inactivation Ions				
CaCl2	10 mM	Extracellular inhibitor of Integrin, binding of Fibronectin	+++	++++
MnCl2	2 mM	Extracellular activator of Integrin, binding of Fibronectin	+++	+
EDTA	0.8 – 1.2 mM	Extracellular divalent cations chelator (Ca^{2+} & Mg^{2+})	+++	+++
BAPTA	60 µM	Intracellular calcium chelator	+	+
Proteases, Integrin ligands, antibodies				
Trypsin	10 µg/ml	Digestion of extracellular proteins	++++	+++++
Thrombin	4 U ml	Digestion of extracellular proteins	++++	++++
RGD peptide	5 – 100 µg/ml	Binding to I-domain (αA domain) of Integrins. Competition of binding to Fibronectin	+++	++++
RAD peptide	5 – 100 µg/ml	Peptide control of RGD	+++	++++
Invasin 197 & 397	5 µg/ml	Binding to Integrin β1 (CD29), induces uptake	+++	++++
Fibronectin	100 - 10 µg/ml	Ligand of Integrin $\beta_1\alpha_5$	+++	++++
Inhibitors membrane signaling & uptake				
Cytochalasin D	1 µg/ml	Cytoskeleton, actin depolymerizing agent	+++	++
Nocodazole	25 nM	Microtubules disrupting agent	+++	++
PMSF	1 mM	Protease inhibitor	+++	-
Wortmannin	10 nM	Inhibitor PIP3 kinase	+++	++++
Monodansylcadaverine	0.3 mM	Disturbs clathrin pathway (clathrin recycling)	-	+
Dynasore	20 µM	Inhibits Dynamin activity	-	**ND**
Calpeptin	280 µM	Serine /threonine protease inhibitor, inhibits calpain proteolytic activity	-	+
U73122	2 µM	Phospholipase C inhibitor	+++	**ND**
Methyl β cyclodextrin	0.1 g/ml	Cholesterol depletion from membranes. Lipid rafts disturbance	(+)[1]	-/+
Inhibitors endosomal functions				
Bafilomycin 1A	1 ng/ml	H^+ ATPase inhibitor. Inhibits acidification of endosomes	++++	-/+*
Ammonium Chloride	20 mM	Raises intravesicular pH	+++	+
Concanamycin A	50 mM	H^+ ATPase inhibitor. Inhibits acidification of endosomes	+++	++
Monensin A	1, 6 & 10µM	Sodium ionophore. Changes membrane potential in endosomes	+++, ++ (10µM)	++
Geldanamycin	50 nM	Hsp90 inhibitor	+++	++

*Synchronized cells
[1]) strain-dependent

3.1.4.3.2 Integrin β1 specific antibodies involved in activation or deactivation status of integrins

In other studies, antibodies that activated or inactivated the integrin β1 subunit were described (Bazzoni et al., 1995; Favre et al., 1992; Lenter et al., 1993; Mould et al., 1997). Antibodies showing these regulatory effects are able to bind to a region on the integrin β1 denominated I-like domain. Two antibodies that bind to this region were tested in our infection conditions. These antibodies are 12G10, which binds to activated integrin β1, as well as AIIB2, that inactivates the integrin β1. Once the antibodies were added to the cells for 30 minutes at 37°C, the infection took place with an MOI 60. In these experiments, no effect was seen in the levels of CagA-P after four hours. Since membrane proteins, including integrins, are constantly recycled; thepossibility that these processes could remove most of the antibodies from the membrane before the infection occurred were very high. In order to diminish membrane recycling, incubation of cells with antibodies was done under 4°C conditions for one hour or at room temperature for 30 minutes with 30 μg of antibodies, followed by infection at 37°C with 5% CO_2 for one or three hours. Again, no effect was seen in the CagA-P levels (data not shown). However, after long exposure of the AGS cells to the AIIB2 antibody, the cells were not able to adhere anymore to the surface of the well, which corresponds to the inactivating effect associated with this antibody, confirm its functionality. The fact that none of these antibodies affected the levels of CagA translocation (see Table 3-1) indicates that the activation status of integrin β1 affected by these antibodies is not relevant for CagA translocation.

3.1.4.3.3 Competition for the integrin $\beta_1\alpha_5$ RGD binding domain

Many proteins are described to interact with integrin heterodimers. Two of them are fibronectin, an extracellular matrix (ECM) component protein, and invasin (Inv), a protein that allows *Yersinia spp.* to adhere and to invade eukaryotic cells in an integrin $\beta_1\alpha_5$ specific way (Dersch and Isberg, 1999). Fibronectin contains an RGD motif, which allows it to bind the I-domain of the α_5 subunit of the integrin $\beta_1\alpha_5$ (Mould et al., 1997). invasin (Inv) contains no RGD motif but an aspartate in position 911 (D911) that presumably functions as the aspartate of the RGD motif of fibronectin (Leong et al., 1995). With the same principle, a RGD-containing peptide and its control (RAD) have been used to establish competition for this domain, inhibiting binding of RGD motifs (and invasin) to the integrin $\beta_1\alpha_5$ heterodimer. To learn if the I-domain found on the alpha subunit of integrin heterodimer is relevant for the

Cag apparatus–integrin interaction, fibronectin, invasin and the RGD and RAD peptides were used in competition experiments. Their effect on CagA phosphorylation was measured. As described in table 3-1, none of them had an effect on the CagA-P levels independent of the infection time (one, two or four hours), the protein concentration (5-100 µg /ml) or the binding conditions (37°C or 4°C). These data can be correlated with the results obtained by the earlier use of inactivating antibodies, which targeted the I-like domain of the β subunit and inactivated the I-domain's capacity of the alpha subunit to bind to RGD containing peptides. It can be concluded from these experiments that the RGD binding domain of the alpha subunit is not relevant for the CagA translocation.

3.2 Integrin β1 interacts with the Cag apparatus.

3.2.1 Integrin β1 interaction with *Helicobacter pylori* is Cag dependent.

3.2.1.1 Integrin $\beta_1\alpha_5$ fluorescence assay

In order to determine if the Cag apparatus interacts directly with integrin β1, a localization of the apparatus on the bacteria was attempted by using a purified, soluble fluorescent labeled integrin $\beta_1\alpha_5$ protein complex (integrin $\beta_1\alpha_5$ $Alexa_{555}$ conjugated). Green Fluorescent Protein (GFP)-expressing *H. pylori* were grown overnight on plates, resuspended and exposed to the integrin $\beta_1\alpha_5$, before they were fixed and analyzed by confocal microscopy. This procedure was performed with bacteria growing on serum plates, as well as with bacteria growing serum-free plates especially developed for these studies. For both cases, with or without serum, integrin $\beta_1\alpha_5$ bound to P12 wild type and P12Δ*cagA* intact bacteria, but not to P12ΔPAI. However, only when bacteria grew on serum-free media, the binding to the recombinant proteins was more specific than to those bacteria that grew on serum plates, as shown for the wild type P12 in figure 3-10. This could be explained by the existence of ECM proteins like fibronectin in the serum plates that adhered to the bacteria while growing. The staining of bacteria using integrin $\beta_1\alpha_5$-$Alexa_{555}$ did show a conserved localization on the bacteria concentrated on spots around the bacterial cell. This concentration was observed only on Cag positive strains and not on the Cag mutant, thus confirming a dependent interaction between the integrin proteins and the Cag apparatus components. Furthermore, ECM proteins are not serving as bridging molecules in this interaction, as serum-free grown bacteria showed a better and defined interaction of the apparatus proteins to integrin β1 protein.

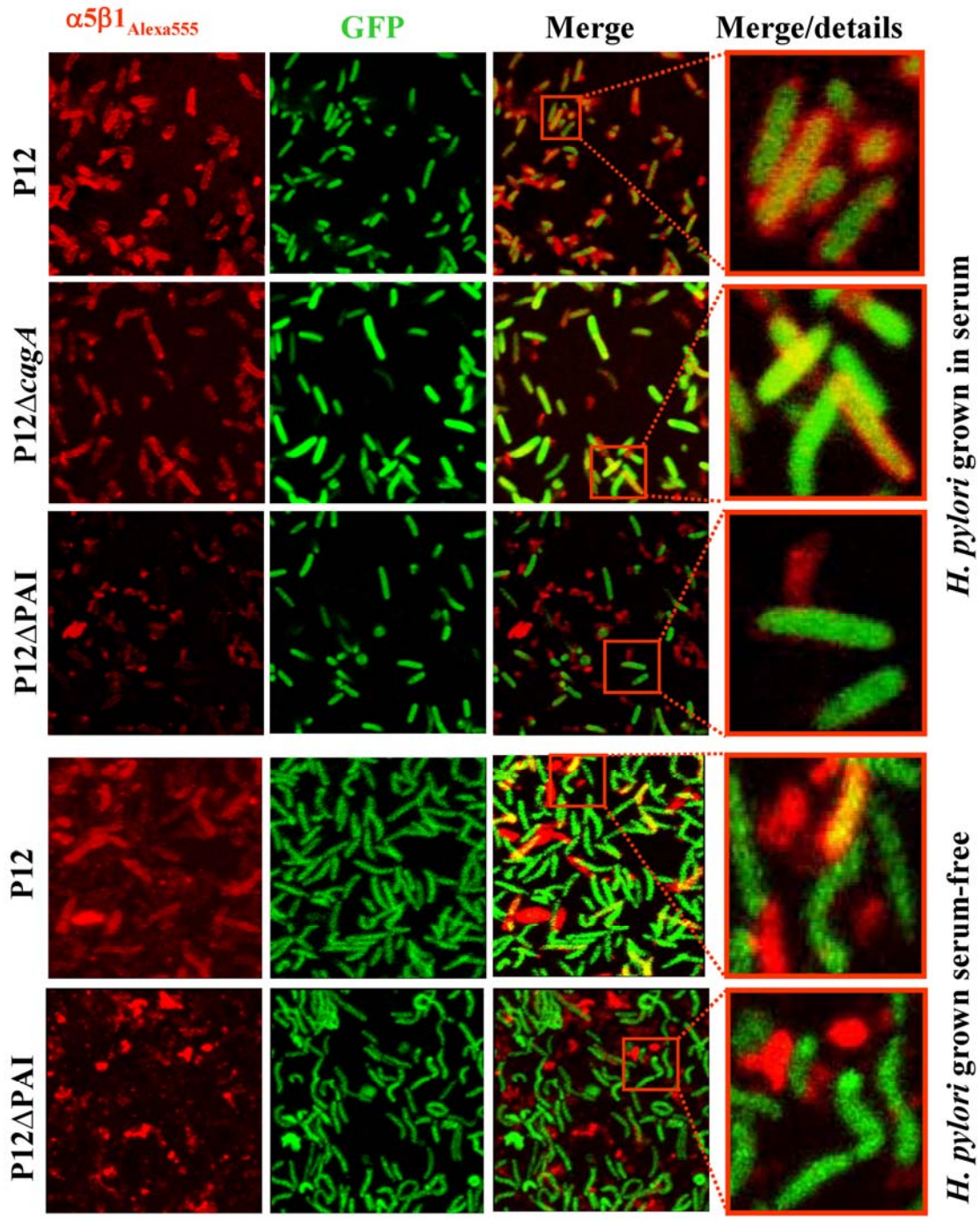


Figure 3-10 Integrin $\beta_1\alpha_5$ binding to P12 CagPAI-dependent

Bacteria expressing GFP (green) were grown on serum or serum-free media, resuspended to OD 0,025 in 1ml of DMEM and exposed to purified integrin $\beta_1\alpha_5$- $Alexa_{555}$ (red). Bacteria were centrifuged, fixed and imaged using a Leica Confocal microscope. Z-stacks with 0,24 µm distance were merged into a single picture. Raw pictures were edited using Metamorph software.

3.2.1.2 Selection of bacteria expressing the Cag apparatus

Not all *H. pylori* bacteria grown on GC–Serum agar plates present the Cag T4SS (Rohde et al., 2003). Under the hypothesis that only *H. pylori* expressing the Cag apparatus were able to

bind integrin $\beta_1\alpha_5$, and taking advantage of the commercial availability of integrin $\beta_1\alpha_5$, a selection of bacteria presenting the Cag apparatus was attempted using integrin $\beta_1\alpha_5$ heterodimer coupled to magnetic beads. The binding of Cag apparatus expressing bacteria with the integrin heterodimer took place under the presence of 10 µg of RGD peptides, to minimize any cross-reaction with the fibronectin that bacteria could have on their surface.

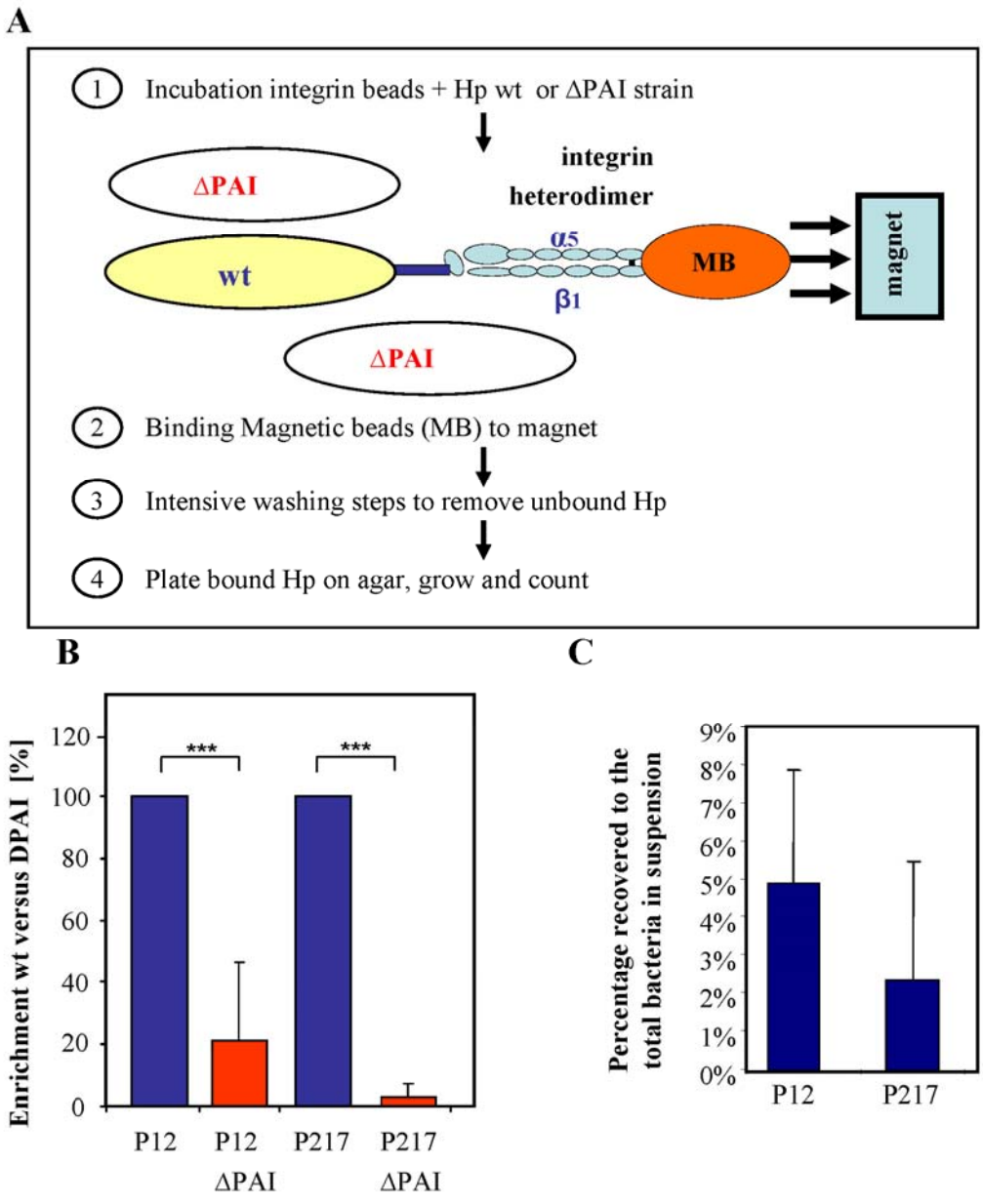


Figure 3-11 Selection of Cag apparatus expressing bacteria using magnetic beads

(A) Scheme of experimental design using integrin $\beta_1\alpha_5$ recombinant protein covalently linked to magnetic beads to select and quantify bacteria expressing the Cag apparatus. wt; wild type. ΔPAI; Mutant lacking the *cag*PAI. (B) Percentage of bacteria recovered using the magnetic beads with recombinant protein. Percentage is relative to wild type strains. Statistical analysis was done using a t-test, n=7. *** P<0.005, ** P<0.01 and *P<0,05. (C) Percentage of recovered wild type strains containing Cag apparatus with magnetic beads in comparison to the initial amount of bacteria in suspension.

After the bacteria were exposed to the coated magnetic beads, the bound bacteria were recovered using a magnet. They were washed, plated and grown for three days in microaerophilic conditions at 37°C in order to quantify the colony forming units (cfu) (see scheme, Figure 3-11A). This procedure was repeated ten times. The results are graphed in figure 3-11B. The interaction of the bacteria with the integrin-coated beads was entirely dependent on the presence of the Cag components, as demonstrated for both strains P12 and P217. As well, it could be estimated that between 2% and 5% of the total amount of bacteria growing on serum plates carry a functional Cag apparatus (Figure 3-11C). Therefore, it can be speculated that the Cag apparati present on bacteria allows them to interact with integrin β1 heterodimers.

3.2.1.3 Integrin β1 co-localizes with H. pylori Cag dependent under infection conditions in vitro

The Cag T4SS binds to the purified integrin $\beta_1\alpha_5$. However, it is necessary to know if the binding happens during *in vitro* infections as well. Using live imaging, a neutral antibody against integrin β1 (Clone 4B7) conjugated to AlexaFluor$_{555}$ was used to label the integrin β1 present on the membrane of living AGS cells. GFP-expressing bacteria, grown on serum-free media, were added at a MOI of 30. By evaluation of life imaging data, co-localization was observed and measured.

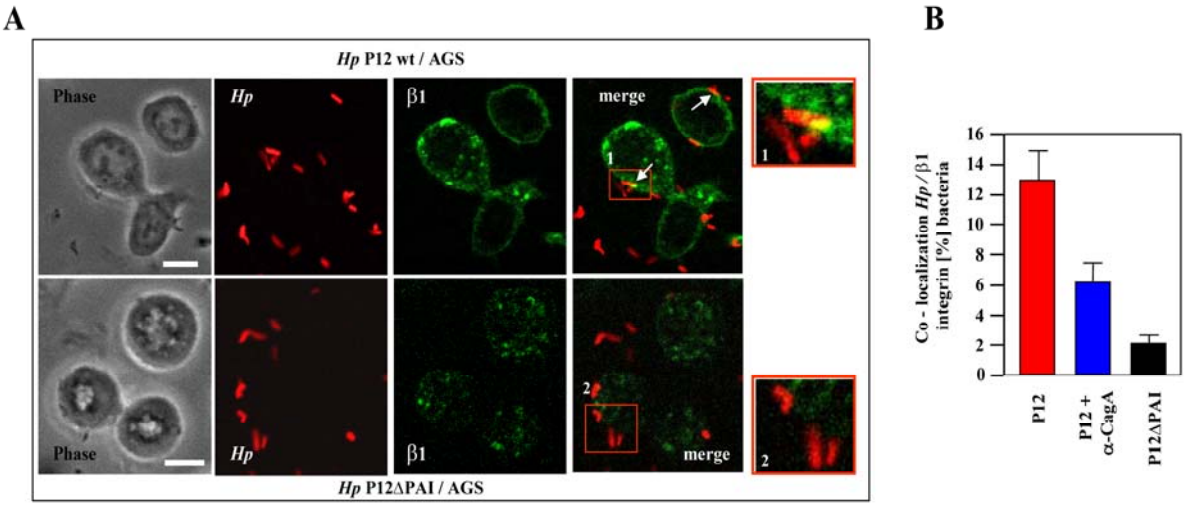


Figure 3-12 Co-localization of integrin β1 with *cagPAI* containing strains during infection of AGS cells

(A) Using an Alexa$_{555}$-conjugated anti-integrin β1 antibody (Clone 4B7), integrin β1 (green) expressed on AGS cells were labeled and observed its co-localization with P12 wild type and P12 Δ*cagPAI* GFP expressing bacteria (red). (B) Co-localization events were counted and graphed in percentage relative to total amount of bacteria counted.

As seen in figure 3-12, only bacteria containing the Cag apparatus components co-localized with the integrin β1 present on the cell. In this way, the relevance of the interaction of integrin β1 with bacteria on a Cag dependent manner under infection conditions was proven.

3.2.2 Integrin β1 interacts directly with CagA, CagY and CagI proteins from the Cag apparatus

The Cag pathogenicity island (*cagPAI*) encodes a T4SS. It is composed of 29 genes, of which most products are essential for the proper functioning of the apparatus. Given the fact that the Cag apparatus interacts with integrin β1, it is important to know which of the proteins encoded in the *cag*PAI are involved in a direct interaction. Two methods were applied in order to find the interaction partners: Yeast two hybrid assay (Y2H) and protein pull down assays using the purified integrin heterodimers proteins ($\beta_1\alpha_5$ and $\beta_1\alpha_1$) covalently attached to magnetic beads.

3.2.2.1 Yeast two hybrid assay (Y2H) (Together with S. Kutter, LMU, Munich)

Until this point, the relevance of integrin β1 has been confirmed by infection of cells from different cell origin: Epithelia (AGS), embryonic epithelial-like (GE11β), embryonic fibroblast-like (GD25β) and hematopoietic (HL-60, Jurkat) cells. Because of their different origin, different sets of alpha subunits are present and interact with the integrin β1 subunit (Boelens et al., 2007; Brakebusch et al., 1997; Hoffmann et al., 2005; Schweighoffer et al., 1994; Sixt et al., 2006). Since the only common factor is the β1 subunit, in frame of the PhD work of Stefan Kutter (Max-von-Pettenkofer Institute, LMU Munich, Germany), a Yeast Two Hybrid (Y2H) assay was performed using the full size extracellular integrin β1 subunit versus all proteins present on the Cag PAI. In figure 3-13, the proteins that gave a positive interaction with integrin β1 are represented.

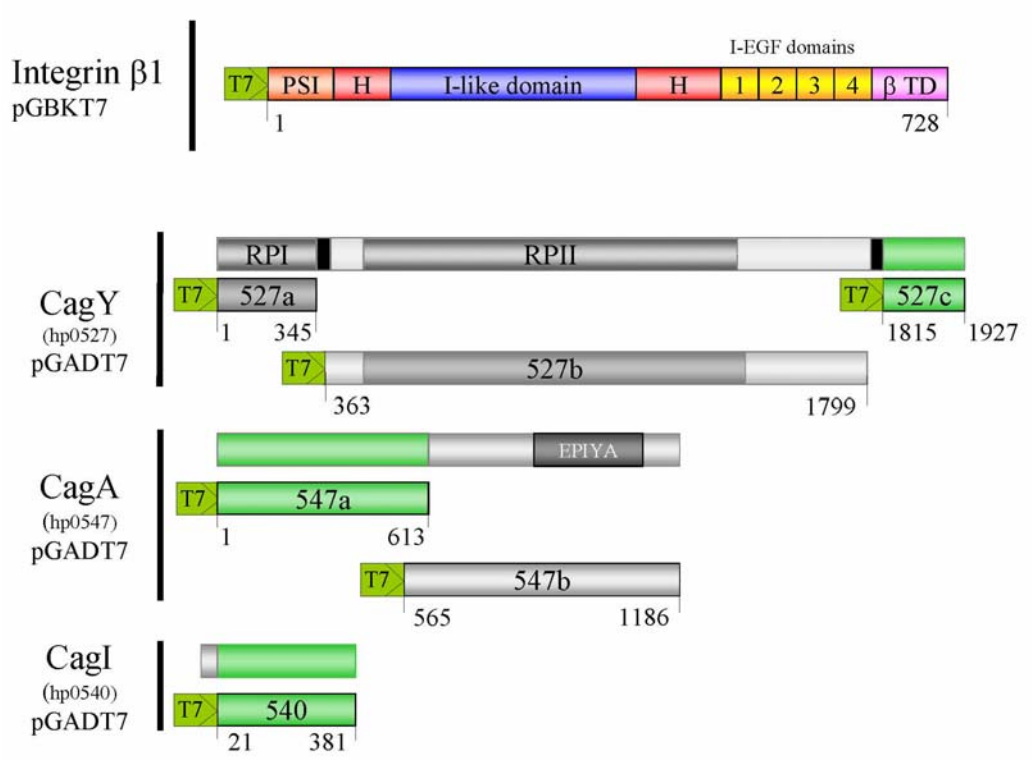


Figure 3-13 Proteins used in Y2H assay

Representation of proteins or protein fragments used in the Y2H assay with integrin β1 extracellular fragment as bait and Cag apparatus proteins as prey. CagA N-terminal and C-terminal halves were expressed independent. The same in the CagY case, with CagYa (HP0527a) being the Repeat Region (RP) I, CagYb (HP0527b) the RPII and CagYc (HP0527c) the region after the second putative transmembrane region. Green segments represent those showing a positive interaction with human integrin β1 extracellular part. pGBKT7, plasmid from bait yeast strain; pGADT7, plasmid from prey yeast strain.

Since CagA and CagY are very large proteins, they were screened in fragments that are considered relevant for the function of the T4SS. For the CagA analysis, it was evaluated in two fragments: the N-terminal part of CagA (547a, Aa 1-613) and the C-terminal part containing the EPIYA motifs (547b, Aa 565-1186). CagY contains three fragments of interest: 527a or CagYa (Aa 1-345), which contain the Repeat Region I (RPI) of the protein; the 527b or CagYb (Aa 363-1799) containing the Repeat Region II (RPII); and the 527c or CagYc (Aa 1815- 1927) with the area of the CagY homologue to the VirB10 protein of *Agrobacterium tumefaciens*. CagI sequence (540, Aa 21-381) was not fragmented, but its signal sequence removed. As seen in figure 3-14, the extracellular domain of integrin β1 showed interactions with only three proteins from the Cag PAI: CagA, CagY and CagI. The results specifically revealed an interaction between the CagA N-terminal and CagY *virB10* homology section (CagYc), and the whole CagI sequence with the integrin β1.

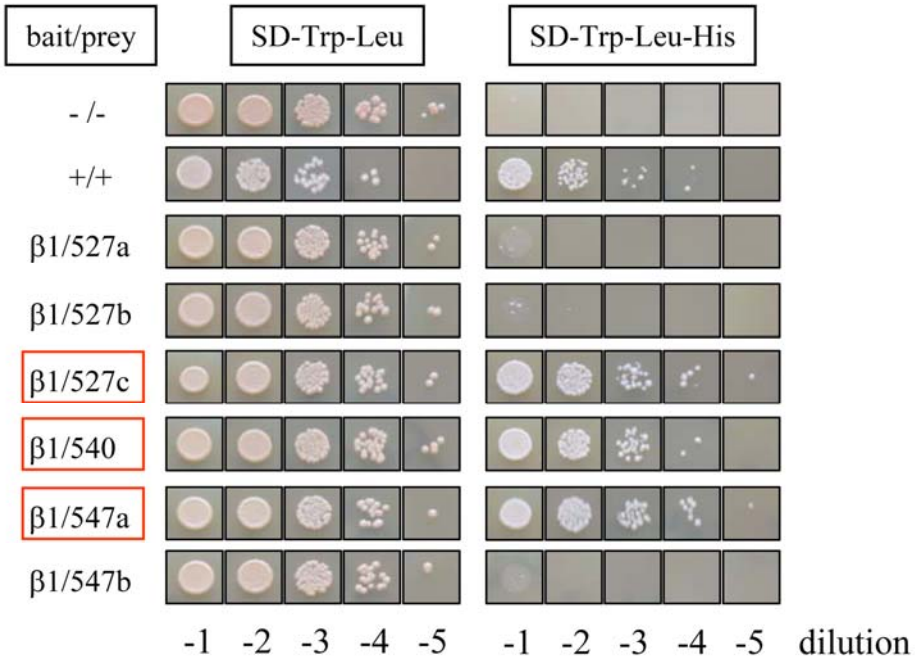


Figure 3-14 Interactions detected in Y2H assay

The growth of yeast shows the interaction between proteins at different dilutions. Control growth of yeast is visualized on SD-Trp-Leu media. Only yeast presenting proteins with a positive interaction, are able to grow in media lacking histidine (SD Trp-Leu-His). Dilutions are to confirm the strength of the interaction. Only CagA N-terminal part (547a), Cag I (540) and CagY C-terminal (527c) shown interaction with integrin β1

With the Y2H assay, it could be shown that only three proteins from the Cag apparatus, CagA N-terminal half, Cag I protein and C-terminal region of CagY, act as interacting partners with the extracellular domain of integrin β1 protein.

3.2.2.2 Validation of Y2H data interactions.

3.2.2.2.1 Protein pull down assays

In order to verify the first data obtained in the Y2H assay, which showed an interaction of CagA, CagY and CagI with the extracellular domains of integrin β1, pull downs were carried out using purified integrin heterodimers integrin $\alpha_1\beta_1$ and $\alpha_5\beta_1$, attached to magnetic beads. With the purpose of obtaining the proteins from the Cag apparatus present on the surface of the bacteria minimizing cross reactions and high background, a new extraction protocol was developed. In this protocol the use of certain detergents was avoided, since they could damage the heterodimer conformation of the integrins. An overview of this protocol can be seen in figure 3-15.

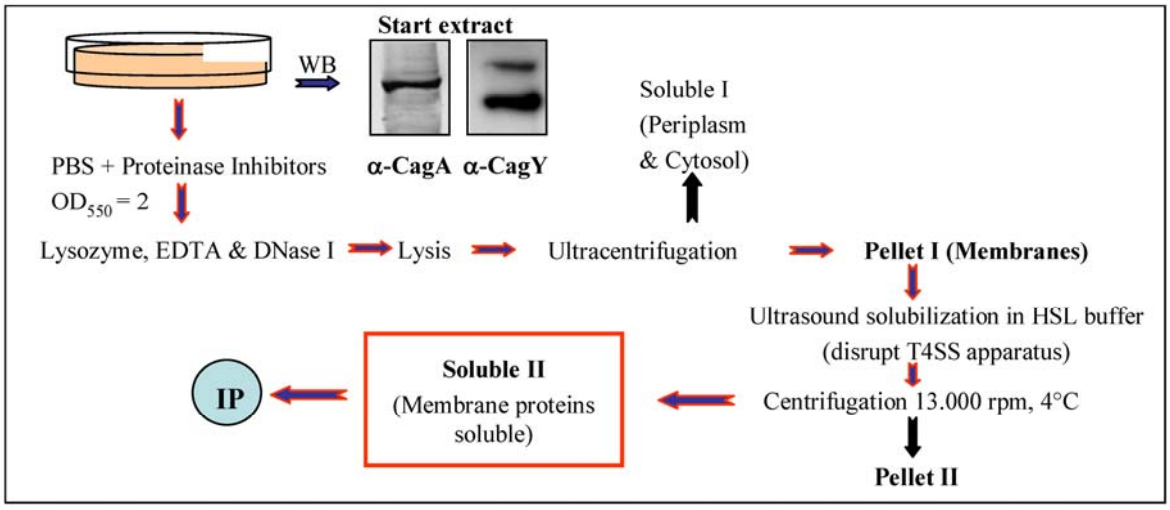


Figure 3-15 Pull down assay using integrin β1 proteins

Scheme of protocol developed to extract Cag apparatus proteins present on the surface of the membrane used to detect those proteins interacting with integrin $\beta_1\alpha_5$ and integrin $\beta_1\alpha_1$ coupled to magnetic beads. HSL, High Salt Lysis Buffer. WB, Western Blot. IP, immunoprecipitation or pull down.

The bacteria were lysed with ultrasonication and the membrane components separated from the cytoplasmic ones. The membrane fraction was exposed to ultrasonication and high salt conditions in an attempt to obtain the proteins attached to the membrane fraction in a soluble form and therefore being able to carry out the pull downs. Once the proteins were solubilized, they were exposed to the integrin proteins on the magnetic beads, and after several highly astringent washing steps, loaded on a gel and immunoblotted to identify which proteins were interacting with the heterodimer.

As observed in figure 3-16, the interaction already seen in the Y2H system of integrin β1 with CagA and CagY was confirmed. Unfortunately, the lack of antibody against CagI did not allow confirming this interaction. However, it was possible to confirm that CagA is able to interact directly with the integrin β1 independently of other proteins of the apparatus, since in P12 the strain lacking the complete island, but expressing CagA in *trans*, CagA showed interaction with the integrin β1.

In these experiments, it has been shown that the alpha subunit does not seem to influence the interaction of the Cag proteins and the β subunit of the integrin. At the same time, it could be confirmed that CagA and CagY are interacting partners with the integrin β1. In the case of CagI, the lack of antibody against it, made it impossible to detect an interaction with integrin β1 through this experiment.

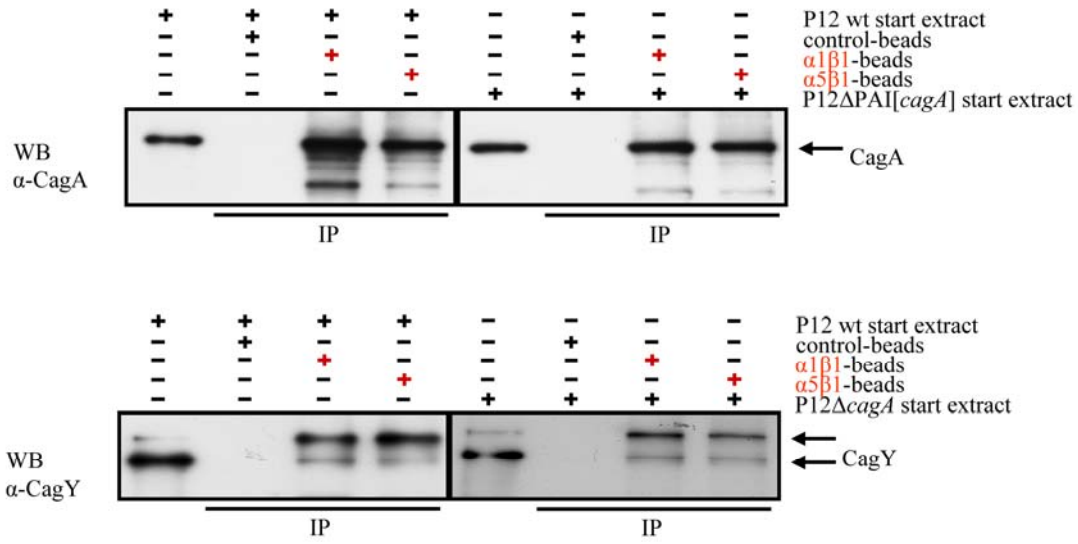


Figure 3-16 Western blot analysis of proteins interacting with integrin β1

Immunoblots detecting CagA and CagY after pull downs using two integrin heterodimers ($\alpha_1\beta_1$ and $\alpha_5\beta_1$) coupled to magnetic beads. Antibodies used to recognize the CagA and CagY were AK257 and AK273 respectively. Initial extract prepared from P12 wild strain and P12 ΔPAI expressing $CagA_{26695}$ protein in *trans* encoded by a plasmid.

3.2.2.2.2 Specific binding of CagA protein to integrin β1

An assay to confirm the importance of the N-terminal half of CagA was attemptedusing antibodies directed against both halves of CagA and testing their capability to inhibit CagA translocation. The purified antibodies were AK268, which is directed against the N-terminal half of CagA, and AK257, which was produced against the C-terminal half of CagA. Result observed during live imaging using bacteria pre-exposed to one of both antibodies revealed a decline in co-localization events with integrin β1 (Figure 3-17B). As seen in figure 3-17A, neither the antibody against the N-terminal region (AK280) nor AK257, which recognizes the C-terminal region, were able to inhibit CagA translocation/ phosphorylation in thestrains tested. Even though a reduction of co-localization of the antibody pre-treated bacteria was seen, it can be concluded that there is no effect on the CagA translocation when the CagA does not colocalize with integrin β1.

It was also necessary to confirm that the interaction between CagA and integrin β1 occurs as well with integrins in their natural form, which is found on the surface of cells. With this intention, a GST fusion protein containing the full size CagA was purified and its binding capacity to cells containing integrin β1 evaluated by flow cytometry. The cells GE11 and a murine fibroblast-like cell line, β1-2-8 (Cells (-)), are cells that do not express integrin β1. Their integrin β1 complemented forms, GE11β and β1-2-8-4 (Cells (+)), were used to define

the integrin β1 specific binding of CagA. In figure 3-17C an evaluation of the CagA binding to integrin β1 containing cells versus their KO forms revealed a specific interaction only with the human form presented by the GE11β cell line, and not with the mouse integrin β1 on the β1-2-8-4 cells (Cells (-)). These results confirm that there is a specific interaction of CagA with the integrin β1 present on cells and it is dependent of the species sequence. It is shown that the effect by the antibodies in live experiments does not correlate with an inhibition of CagA-P in infection conditions as well.

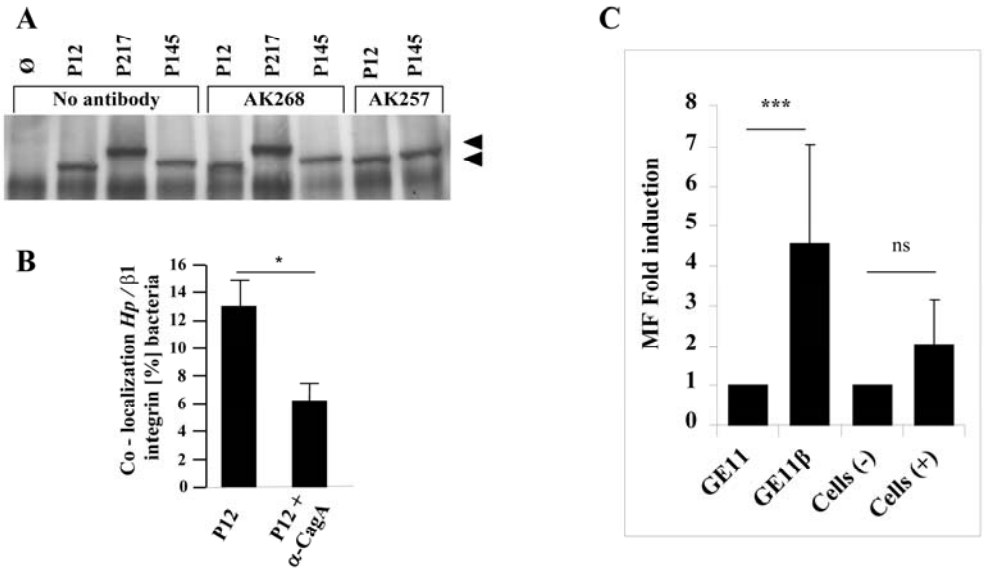


Figure 3-17 CagA Antibody effect on CagA translocation and CagA binding to integrin β1

(A) Effect of antibodies recognizing N- and C-terminal regions of CagA on CagA translocation. Bacteria were exposed to 12 µg antibody before infection.(B) Co-localization of antibody-labeled CagA on bacteria with antibody-labeled integrin β1 on AGS cells during infection (C) Specific binding of GST-CagA to cells in an integrin β1 dependent manner. Binding tested on cells KO integrin (GE11 and Cells (-)) and complemented forms (GE11β and Cells (+)). GST-CagA binding was evaluated through immunofluorescence and flow cytometry after immunodetection of the GST tag. Statistical significance was evaluated using a t-Test, n = 8, *P<0,05, *** P<0,005, ns, no significance.

3.2.2.2.3 The C-terminal fragment of CagY (CagYc) interacts with integrin β1

Since in the Y2H assay a specific interaction of C-terminal fragment of CagY (CagYc) was shown, and at the same time integrin β1 pull downs confirmed an interaction with CagY, it was necessary to confirm the specificity of this interaction with the CagYc using a GST-fusion protein. Using GST proteins coupled to beads as control, pull downs were done using the GST-CagYc protein and the purified integrin heterodimer. As can be observed in figure 3-18A, only beads containing CagYc showed a binding to integrin β1.

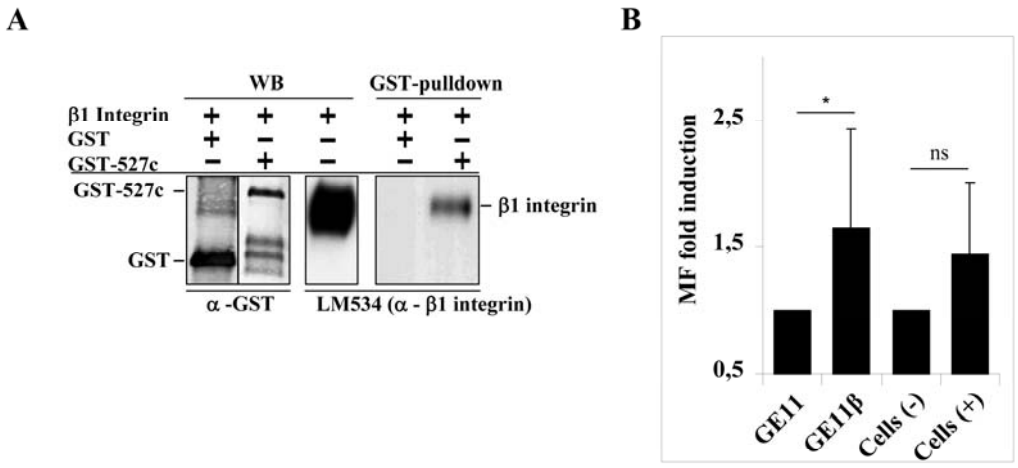


Figure 3-18 CagYc interaction with integrin β1

(A) Immunodetection of fusion protein and its interaction with integrin β1 by Western blot. First three lanes show the GST and GST-CagYc proteins using, anti-GST antibody, and the detection of integrin β1 with the antibody LM534. Last two lanes shows the immunodetection of the integrin β1 precipitated with the GST and GST-CagY. All samples were prepared in non-reducing conditions. (B) Binding of GST-CagYc fusion proteins to cells with or without integrin β1 expression. Binding tested on cells KO integrin (GE11 and Cells (-)) and complemented forms (GE11β and Cells (+)). Fold induction of the mean fluorescence detected by flow cytometry. $Alexa_{488}$ conjugated anti-mouse antibodies detected the anti-GST antibody attached to the GST-CagYc bound to cells. Fold induction is relative to the mean fluorescence of the cells treated with primary and secondary antibodies only. Statistical significance was evaluated using a t-Test. n = 8. * P<0,05.

Similar to the binding of CagA to cells in an integrin β1 dependent manner, also binding of GST-CagYc fusion protein to cells in an integrin β1 dependent manner was measured using flow cytometry analysis. As shown in figure 3-18B, GST-CagYc protein shows binding to the cells in an integrin β1-dependent way. The GST-CagYc fusion protein shows a preference in binding to the human form of the integrin β1 as well. These data confirm that the interaction of CagY with integrin β1 takes place in the C-terminal region of CagY.

3.2.2.2.4 CagI protein interacts with integrin β1

The Y2H assays showed a positive interaction between integrin β1 and CagI. During experiments using integrin β1 for pull down assays, CagI's presence could not be proven because of the lack of an antibody against it. Therefore, a GST fusion protein with the same sequence of CagI as used for the Y2H assays was produced. The GST tag is N-terminal attached, and it was used to detect the binding of CagI to cells only when these expressed integrin β1 on the surface. Using the same method as with the GST-CagA and GST-CagYc, GST-CagI was exposed to cells with or without integrin β1: GE11, GE11β, β1-2-8 (Cells (-)) and β1-2-8-4 (Cells (+)). Using antibodies to detect the GST tagbinding of the CagI was

determined by flow cytometry. As seen in figure 3-19B, CagI binds to cells in an integrin β1 dependent manner GST-CagI showed a higher specificity and affinity to cells expressing the human form (GE11β) of the integrin β1 in comparison to the mouse form present in cell line β1-2-8-4 (Cells (+)) to which CagI bonds independent of integrin β1.

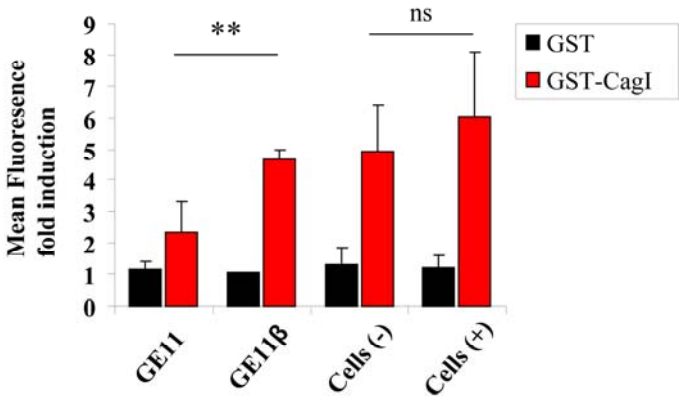


Figure 3-19 GST-CagI binding to integrin β1 cells.

(A) Binding of GST-CagI protein (red bars) to cells without integrin β1 (GE11 and Cells (-) (β1-2-8)) and with it (GE11β and Cells (+) (β1-2-8-4)). For both graphics, the mean fluorescence fold induction is in comparison to the fluorescence of control cells without a GST fusion protein but exposed to primary (anti-GST) and secondary (Anti-Mouse $Alexa_{488}$) antibodies. Data represent the average and standard deviation from three independent experiments. Statistical significance was evaluated using a t-Test, n = 3. *P<0,05, ** P<0,01; ns, no significance.

3.2.2.3 Position of CagA on the tip of the apparatus correlates with its relevance as interacting partner with integrin β1

Given the fact that CagY has already been identified on the surface of the Cag apparatus in infection conditions, it was decided to look at the position of CagA on the Cag apparatus using immunofluorescence with $AlexaFluor_{555}$ labeled AK257 antibodies, and immunodetection in electron microscopy (collaboration with Dr. Manfred Rohde, Helmholtz Center for Infection Research (HZI) Braunschweig). As seen in figure 3-20, data in immunofluorescence shows that CagA can be found on the surface of the bacteria. In the case of electron microscopy, it was confirmed that CagA is localized at the tip of the Cag apparatus, putting it in the right place for an interaction with the integrin β1.

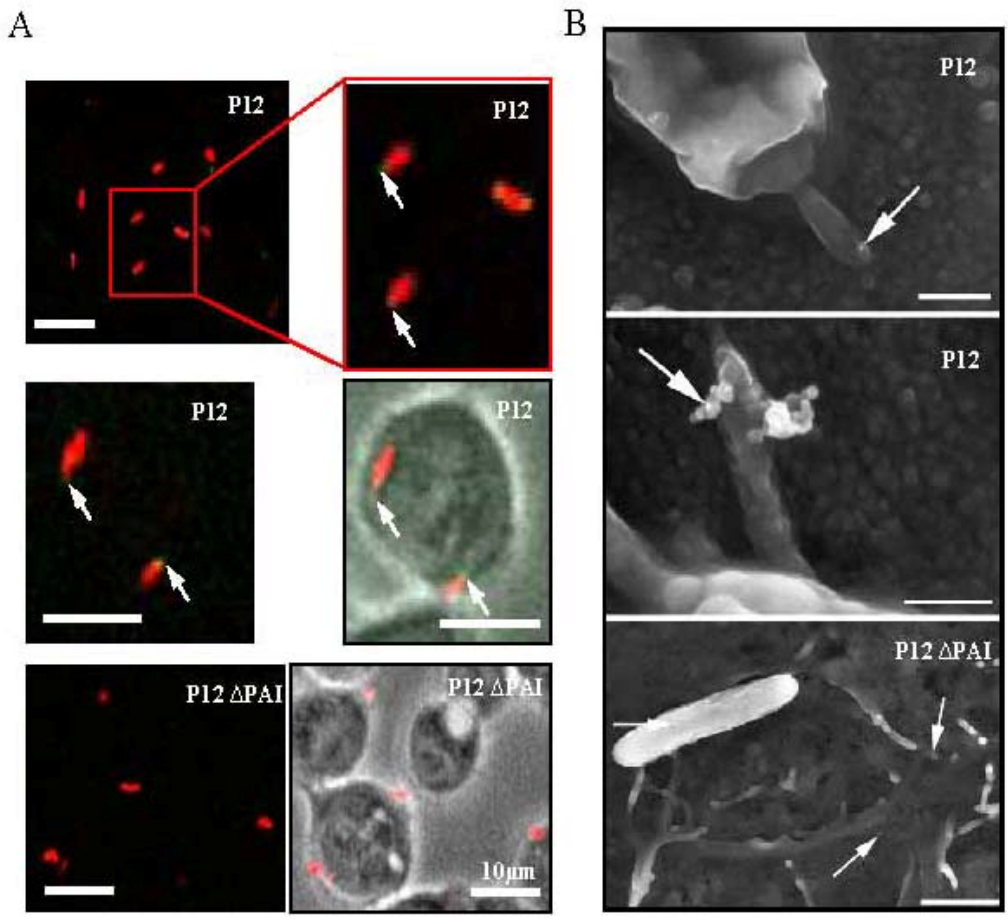


Figure 3-20 Localization of CagA on the apparatus

(A) Immunodetection of CagA in infection conditions. CagA (green) was detected using a fluorescent-labeled anti-CagA antibody (AK257-Alexa$_{647}$). Bacteria (red) produce GFP protein encoded in a pHel4 plasmid. (B) EM pictures showing immunogold detected CagA positioned on the tip of the apparatus.

3.2.3 Relevant integrin β1 domains for the interaction with CagA, CagY and CagI proteins

The integrin β1 subunit has been studied extensively. Its crystal structure and functional domains are well known, and some of the domains functions have been elucidated by the use of specific anti-integrin β1 antibodies (Calderwood, 2004; Takagi and Springer, 2002; Wegener et al., 2007). A better evaluation of the relevant domains of the integrin β1 interacting with the Cag proteins can give a better understanding of the mechanisms involved in CagA translocation.

3.2.3.1 Yeast-two-Hybrid Data (In colaboration with C. Ertl, LMU, Munich)

Since the amount of possible interacting partners of the T4SS with integrin β1 was restricted to the CagA N-terminal half (CagAa), the CagY C-terminal part (CagYc) and CagI, an exact evaluation of which domains from the integrin β1 are interacting with these Cag proteins was important. This information can give an idea of how this interaction takes place for the CagA translocation into the cytoplasm of the eukaryotic cell.

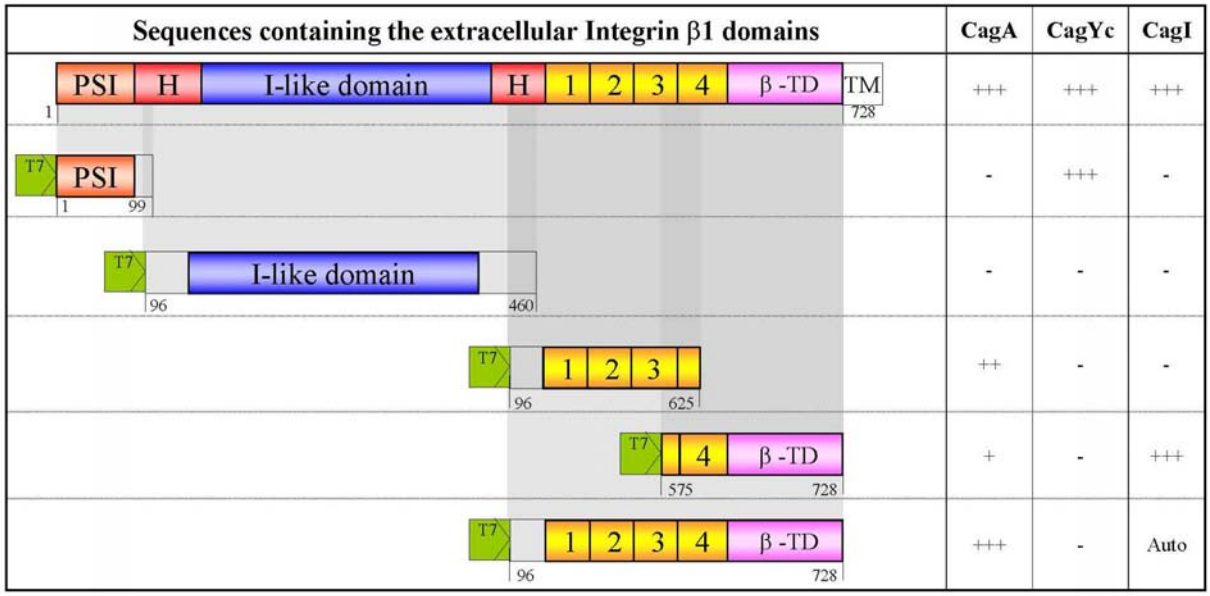


Figure 3-21 Y2H data for interaction of CagPAI proteins with integrin β1 domains

Fragments of integrin β1 used in the Y2H screen. They were used to evaluate the binding specificity of CagA (N-terminal, CagAa), CagYc and CagI to the integrin β1. Results of the screen are described here. Positive reactions and their intensity are represented by (+). Negative interactions by (-), and *Auto* represents autoreactivity or unspecific growth of the yeast in the assay.

The screen was made in collaboration with Claudia Ertl (Max-von-Pettenkofer Institute, LMU, Munich). The fragments used in this screen contained the integrin β1 domains PSI (Aa 1-99), I-like domain (Aa 96-460), EGF domain (1-4) (Aa 410-625), β tail domain (βTD) (Aa 575-728) and a EGF (1-4)-βTD (Aa 410 -728). All of these domains were screened for interactions with CagA (N-terminal), CagYc and CagI protein in the Y2H assay.

From this screen, interaction between CagYc and PSI was established. CagA (N-terminal) gave a weak interaction with EGF (1-4) and strong interaction with the EGF (1-4)-βTD fragment. In the case of CagI, its interaction was related to the βTD domain fragment (See Figure 3-21).

3.2.3.2 Validation of Y2H interactions

The previous results had to be confirmed with other methods and their relevance in the Cag apparatus functionality had to be evaluated. This was approached by evaluation of effects of specific antibodies targeting the integrin β1 domains in CagA translocation, as well as through binding assays under different conditions.

3.2.3.2.1 EGF repeat domain involved in CagA translocation

Knowing that CagA possibly interacts with a region between the EGF (1-4) domain and the βTD of integrin β1, and CagI with the βTD, antibodies directed against these regions were used to inhibit CagA translocation and phosphorylation. As shown in figure 3-22B, CagA phosphorylation is blocked only with the antibody 9EG7. 9EG7 is an antibody that binds to human integrin β1 at a domain made accessible in the presence of Mn^{2+} (Bazzoni et al., 1995). The effect on blocking the translocation is directly proportional to its capacity to bind to the integrin β1, as seen after a one-hour infection in conditions with and without Mn^{2+}. None of the antibodies K20 and LM534 affected the CagA phosphorylation (Figure 3-22B), even though they partially overlap the binding epitope recognized by 9EG7 (Figure 3-22A). As seen here, the AIIB2 antibody does not have an effect on CagA phosphorylation. This data is in agreement with the fact that the I-like domain of the β1 integrin showed no interaction with any of the Cag proteins tested. All antibodies were able to bind to the AGS cells (Figure 3-22C), and the already described effect of Mn^{2+} for 9EG7 was observed as well.

These results lead to the possibility that competition between the apparatus proteins (CagA and CagI) and the antibody could be the cause of the inhibition. To assess this hypothesis, binding assays using 9EG7 to inhibit GST-CagA, GST-CagI and GST-CagYc binding to integrin β1 expressing cells (AGS) were analyzed using flow cytometry. As shown in figure 3-23A, the binding of 9EG7 did not reduce the binding of any of the fusion proteins to the AGS cells. These results are independent from the presence of Mn^{2+} in the binding assays. Thus, 9EG7 antibody does not inhibit the binding of GST-CagA, GST-CagYc or GST-CagI to integrin β1.

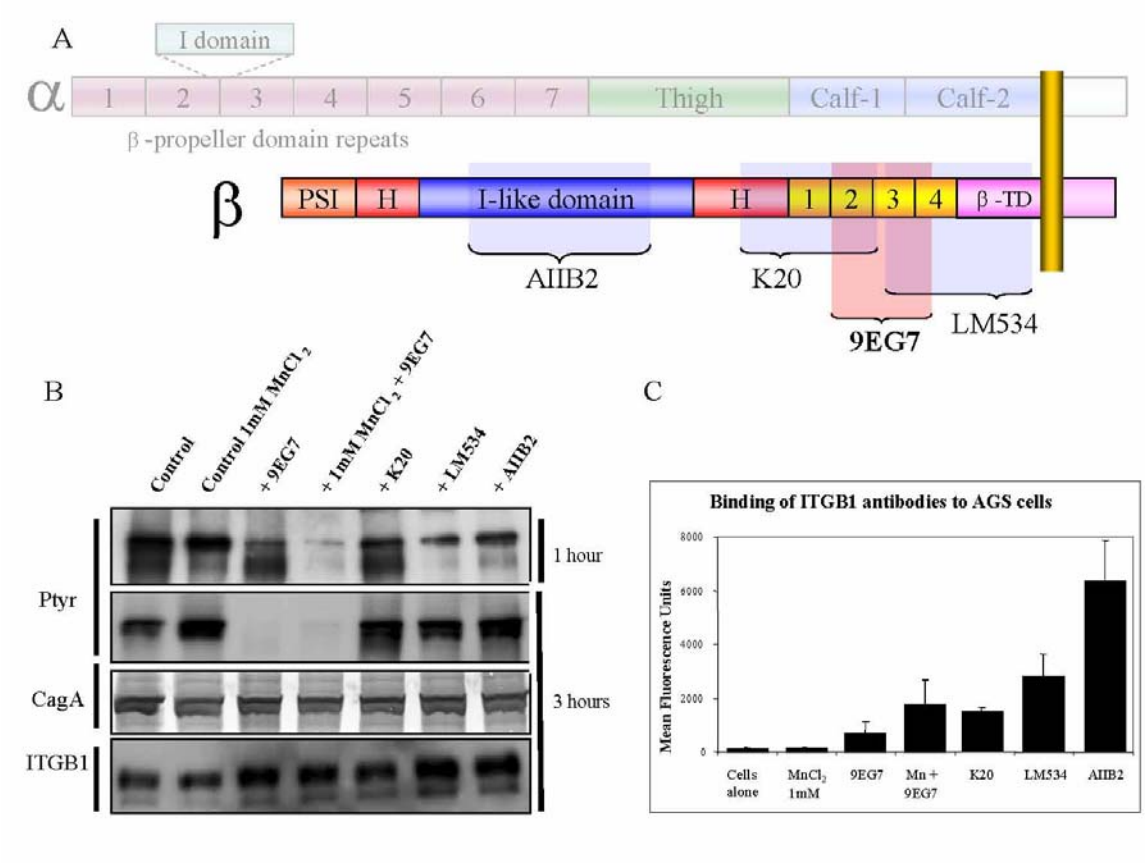


Figure 3-22 Evaluation of integrin β1 domains relevance in CagA phosphorylation

(A) Localization of epitopes recognized by antibodies used to evaluate the relevance of integrin β1 domains in CagA translocation. (B) Immunodetection of CagA phosphorylation after 1 and 3 hours of infection of cells pre-exposed for 30 minutes to anti-integrin β1 antibodies (30 µg/ml). The CagA protein and integrin β1 were detected using antibodies AK257 and LM534, respectively. (C) Evaluation of binding capacity of antibodies to AGS cells during the pre-incubation period before infection was done using immunofluorescence and analysis through flow cytometry.

3.2.3.2.2 CagYc could be interacting with the PSI domain

Since binding of CagYc was mapped to the PSI domain, and structural studies from integrins show that the PSI domain is exposed when integrins are activated, binding assays to evaluate the binding of CagY in activated conformation were set. To achieve an activated form of the integrin β1 on the cells, Mn^{2+} was used. The results are illustrated in figures 3-23B and 3-23C. The binding of the fusion proteins was evaluated under free bivalent cations conditions. The amount of $MnCl_2$ used to activate integrin β1 was 1- 2 mM. Cells were exposed to fusion protein and left to bind under protease inhibitor solution for one hour at 4°C. Binding of the proteins was detected by immunodetection of the GST fusion protein moiety and analyzed by flow cytometry. Only GST-CagYc, not GST-CagA or GST-CagI, showed an increased tendency to bind in presence of $MnCl_2$, which supports the idea that the binding of the CagY through its C-terminal part to the integrin β1 occurs in the PSI domain.

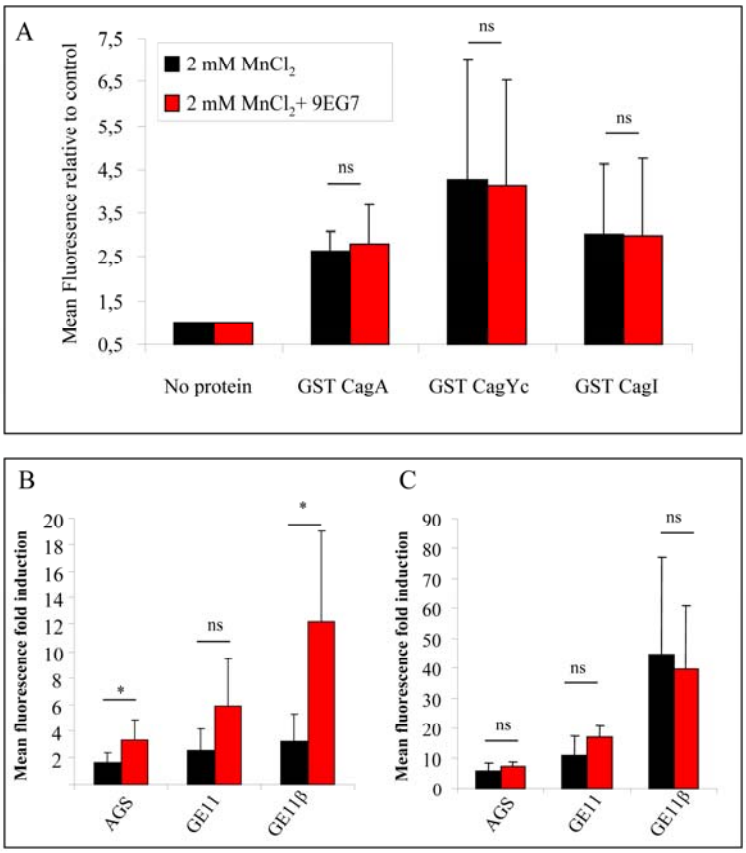


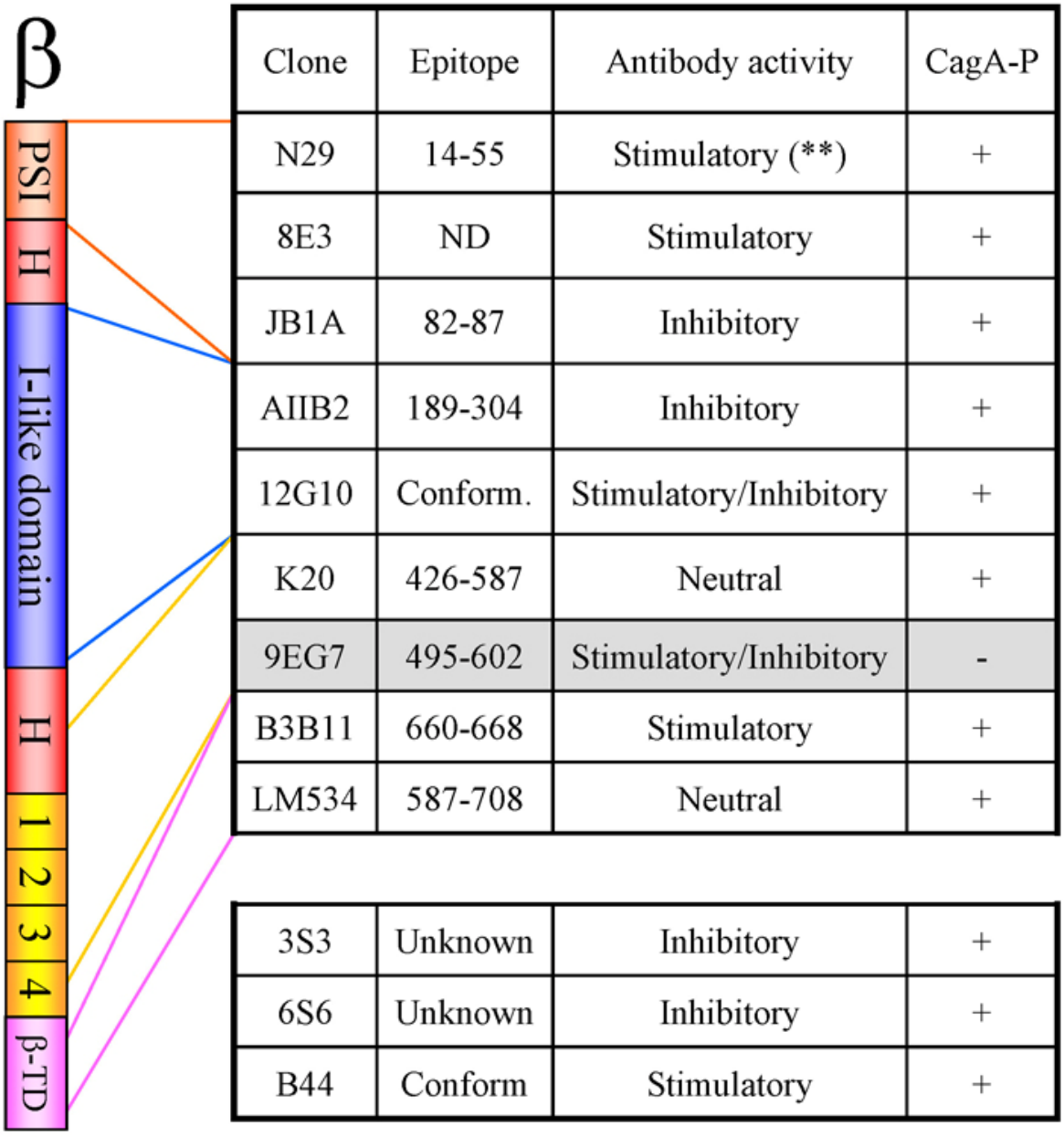
Figure 3-23 Effect of 9EG7 antibody and $MnCl_2$ on binding fusion proteins

(A) After pre-incubation of AGS cells with 9EG7 antibody for 30 minutes, GST-CagA, GST-CagYc and GST-CagI protein binding was evaluated by immunostaining and flow cytometry. (B) Effect of 1mM $MnCl_2$ on binding of GST-CagY and (C) GST-CagA protein. Dark bars indicate the binding of the fusion protein without $MnCl_2$, red bars indicate the binding of fusion proteins in presence of 2 mM $MnCl_2$. The mean fluorescence fold induction is relative to the control cells exposed to anti-GST and anti-mouse $Alexa_{488}$ antibodies. Statistical data analysis using t-Test. ** P< 0,01; ns, No significance

3.2.3.3 Relevance of integrin β1 domains in CagA translocation using antibodies

Using antibodies binding to different regions of the integrin β1 proteins, it was expected to elucidate in detail other essential mechanism involved in the CagA translocation. As depicted in figure 3-24, no other epitopes, recognized by the set of monoclonal antibodies, are relevant in CagA translocation, as no changes on the CagA phosphorylation levels were observed after infection of synchronized AGS cells pre-incubated with different antibodies. The data relevant to the antibodies' activity on the binding capacity of integrin refer to published data by Wilkins, JA (Wilkins et al., 1996; Clones N29, B2B11, B44), Mould, AP (Mould et al., 1997; Clones 8E3, 12G10), Stupack, DG (Wilkins et al., 1991; Clone JB1A), Hall, D.E. (Hall et al., 1990; Clone AIIB2), Ni, H. (Ni et al., 1998; Ni and Wilkins, 1998; Clone K20),

Bazzoni, G. (Bazzoni et al., 1995; Clone 9EG7), Takada, Y. (Takada and Puzon, 1993; Clone LM534), Gao, J.X. (Gao et al., 1995; Gao and Issekutz, 1995; Clone 3S3) and Gonzales, A.M. (Gonzalez et al., 2002; Clone 6S6).



Clone	Epitope	Antibody activity	CagA-P
N29	14-55	Stimulatory (**)	+
8E3	ND	Stimulatory	+
JB1A	82-87	Inhibitory	+
AIIB2	189-304	Inhibitory	+
12G10	Conform.	Stimulatory/Inhibitory	+
K20	426-587	Neutral	+
9EG7	495-602	Stimulatory/Inhibitory	-
B3B11	660-668	Stimulatory	+
LM534	587-708	Neutral	+
3S3	Unknown	Inhibitory	+
6S6	Unknown	Inhibitory	+
B44	Conform	Stimulatory	+

Figure 3-24 Summary of effect of antibodies against integrin β1 on CagA phosphorylation

Antibodies tested to evaluate their effect on CagA phosphorylation. Recognized antibodies' epitopes are described here. A linear epitope has not been defined for all antibodies. *Conform*, for those antibodies recognizing epitopes known to be available under a certain activation status of the integrin heterodimer. *Neutral* defined as not observed any effect on binding capacity of the integrins. *Inhibitory* defines antibodies that diminish the binding capacity of the integrin heterodimers. *Stimulatory* are antibodies that enhance the binding to their substrate. Positive or negative representation of phosphorylated CagA refers to phosphorylation of P12 CagA. Antibodies used in a concentration of 30 µg/ml to pre-treat synchronized cells for 30 minutes, after 30 minutes from the addition of serum containing media. Infection was done for one hour with MOI 60. Data summarizes the results of at least two independent experiments

3.3 Integrin β1 and the induction of IL-8 production via Cag

The Cag apparatus does not only translocate the CagA protein into the cell cytoplasm. It is as well responsible for the induction of cytokine production by human cells, like Interleukin 8 (IL-8) and RANTES (Innocenti et al., 2002; Nozawa et al., 2004). This effect is not seen in mouse cells (personal observation and Ferrero et al., 2008). In the previous part of this thesis, it has been shown that the integrin β1 is essential for the CagA translocation and phosphorylation. At the same time that CagA phosphorylation was assessed, the effects on IL-8 induction were measured and analyzed.

3.3.1 Dynamics of IL-8 production

In order to establish if the IL-8 induction produced by the T4SS of *H. pylori* is possible through a receptor interaction, the dynamics of IL-8 secretion inAGS were studied using two different stimuli. Tumor Necrosis Factor alpha (TNFα) and Phorbol Myristate Acetate (PMA). TNFα induces IL-8 production by binding to its receptor, the TNFR. PMA is a cell membrane permeable PKC activator that can induce IL-8 production. The TNFα was used as an example for IL-8 induction via a receptor, while PMA's effect on IL-8 represents a receptor independent stimulus. The experiments were done with cells that have not been synchronized. Two different concentrations were tested for TNFα (50 and 100 pg) and PMA (50 and 5 ng/ml). Supernatants were collected in time intervals of 10 minutes and IL-8 was measured through a sandwich ELISA. As seen in figure 3-25, both stimuli show an increment on IL-8 production at 50 minutes, TNFα stimulated cells being the first ones to increase exponentially (Figure 3-25A). In the case of PMA, which does not need a receptor for IL-8 induction, the IL-8 induction presents a small plateau of production between the first measurement at 50 minutes and the exponential increment at 90 minutes. It seems that the IL-8 production after the receptor activation mediated by TNFα leads to this plateau for a shorter time (between the 70 to 90 minute time points, 20 minutes) as seen in the IL-8 dynamics of PMA. In the case of a higher concentration of TNFα, this plateau did almost not exist.

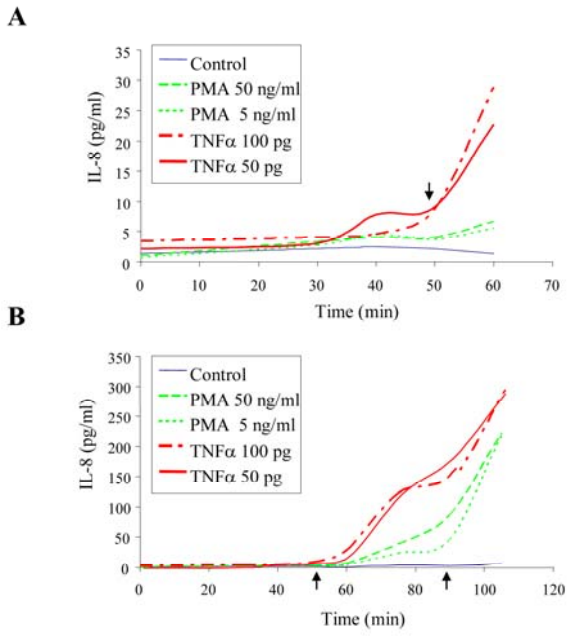


Figure 3-25 IL-8 dynamics with TNFα and PMA

IL-8 concentrations (pg/ml) at different time points after stimulation of AGS cells with Tumor Necrosis Factor α (TNFα, 50 and 100 pg) and Phorbol Myristate Acetate (PMA, 5 and 50 ng/ml). Arrows indicate time points where differences on release of IL-8 were noticed. (A) Time lapse 0 - 60 minutes. (B) Time lapse 0 - 110 minutes. Curves represent data from at least two independent experiments

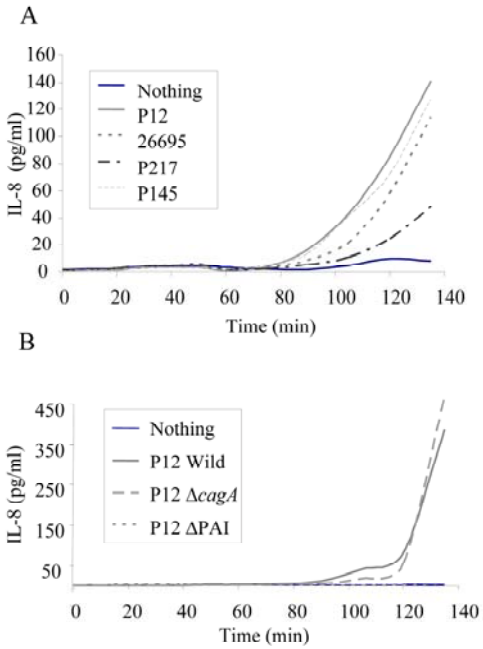


Figure 3-26 IL-8 dynamics of AGS cells induced by the Cag apparatus of *Helicobacter pylori*

IL-8 concentrations (pg/ml) were determined at different time points after stimulation of AGS cells with (A) Three different wild type strains containing a functional Cag apparatus, and (B) P12 wild type and mutants of the Cag apparatus, P12Δc*agA* and P12ΔPAI. All infections were done using an MOI 60. Curves are representative from at least two independent experiments

IL-8 induction dynamics via Cag apparatus, were evaluated using four different strains in infections with MOI 60 of AGS cells. In figure 3-26A, it can be appreciated that IL-8 induction dynamics are dependent of the strain used. Not shown here is that the IL-8 induction was also dependent of the fitness of the bacteria. In general, it can be said that the IL-8 production induced by *H. pylori* in a CagPAI-dependent manner starts approx. 60 to 90 minutes after infection (Figure 3-26B). In the case of IL-8 induction via Cag, no pronounced plateau was observed even in the cases were CagA was not present.

3.3.2 Effect of divalent cations on IL-8 induction via Cag apparatus.

Since the activation of integrin β1 through Mn^{2+} does increase the CagA translocation levels, the effects of $CaCl_2$ and $MnCl_2$ on IL-8 induction were evaluated. Non-synchronized cells were pre-incubated with normal RPMI media, or additionally 2 mM $MnCl_2$ or 3 mM $CaCl_2$ media. The infection took place in standard conditions and the supernatants were collected to measure its IL-8 content using an sandwich ELISA. *H. pylori* P12 strains containing mutations lacking the *cagA* and the entire *cag*PAI were used. It has been shown that CagA alone is responsible for part of the IL-8 induction associated with the Cag T4SS (Fischer et al., 2001). As seen in figure 3-27A, $MnCl_2$ reduces the IL-8 induction effect up to 50% in the case of the wild type. This reduction can be assumed not to have an effect on the CagA translocation, since CagA is translocated and phosphorylated in 1 mM $MnCl_2$ solution. The difference already seen in IL-8 induction for the wild type is as well observed during infections with P12Δ*cagA* in presence of $MnCl_2$. In the case of $CaCl_2$, there is no effect caused by its presence on the IL-8 production.

Consequently, only activation of $MnCl_2$ affects the IL-8 induction via the Cag apparatus in infection of AGS cells. Additionally, this effect is not CagA dependent but Cag apparatus dependent. For calcium, no effect can be concluded from the inactivation of integrins by calcium on the extracellular environment. However, in the case of intracellular calcium, BAPTA inhibits IL-8 induction making it undetectable with our assay (depicted later in figure 3-35), consistent with the inhibition of CagA phosphorylation seen before.

3.3.3 Relevance of RGD binding domain in IL-8 induction

The RGD binding domain of the integrin heterodimers showed not to be relevant for CagA translocation. At the same time, their effect on IL-8 was measured and evaluated. The pre-treatment of cells for 30 minutes with fibronectin, invasin, RGD peptide and its RAD

counterpart, did not show a significant change on the IL-8 induction, as graphed in figure 3-27B and summarized in Table 3-1.

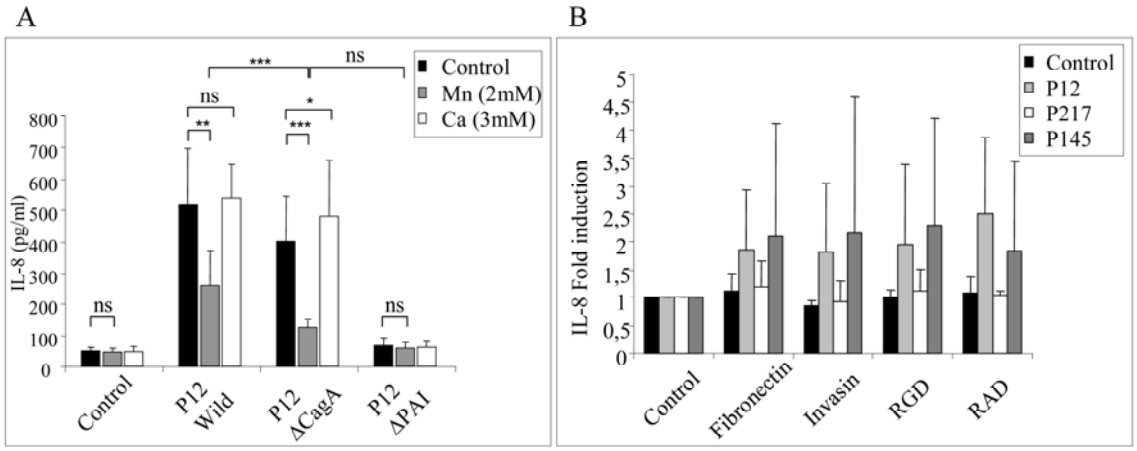


Figure 3-27 Effects of bivalent cations and RGD binding domain competition on IL-8 induction

(A) Effect of calcium (Ca) and manganese (Mn) in the IL-8 induction in a Cag apparatus dependent manner. IL-8 concentrations in pg/ml. (B) Evaluation of integrin $\alpha_5\beta_1$ ligands in IL-8 induction via Cag apparatus. Fold induction is relative to control infections. Strains used were P12, P217 and P145 for 4-hour infections with a MOI 60. Statistical analysis using t-Test. * P<0.05, ** P<0,01, ***P< 0,005. ns, no significance

3.3.4 Effects of ILK signaling in IL-8 induction

In order to determine if integrin β1 signaling is involved in the IL-8 production, the effects of knocking down ILK protein through siRNA were measured during induction of IL-8 by bacteria containing the Cag apparatus. The integrin-linked kinase (ILK) has been described to interact directly with the integrin β1 cytoplasmic domain upon activation of the integrin β1. As shown before, reduction to up to 70% of the ILK levels in the cell are not disturbing the levels of phosphorylated CagA. In the same conditions, IL-8 induction differences were analyzed using *H. pylori* P12 wild type, P12Δ*cagA* and P12 ΔPAI. These mutants were used in order to differentiate between IL-8 induction by the Cag apparatus from the one induced by CagA alone. CagA alone is responsible for part of the IL-8 induction by the Cag apparatus. No change was expected in the induction on IL-8 in cells were ILK protein was reduced, since CagA could be successfully translocated/phosphorylated in these cells regardless of low ILK- levels. As shown in figure 3-28, reduction of ILK levels in AGS cells caused a lost of their sensitivity to induction of IL-8 via the Cag apparatus. As expected, in control cells there is a significant difference between the IL-8 induced by P12 wild type and P12 Δ*cagPAI*, as well as between P12 Δ*cagA* and P12 Δ*cagPAI*.

Thus, ILK signaling is not required for CagA translocation, but it is necessary for IL-8 induction via the Cag apparatus. These results in conjunction with the effects of the bivalent cations Mn^{2+} on IL-8, indicate a possible role of integrin β1 on the IL-8 induction

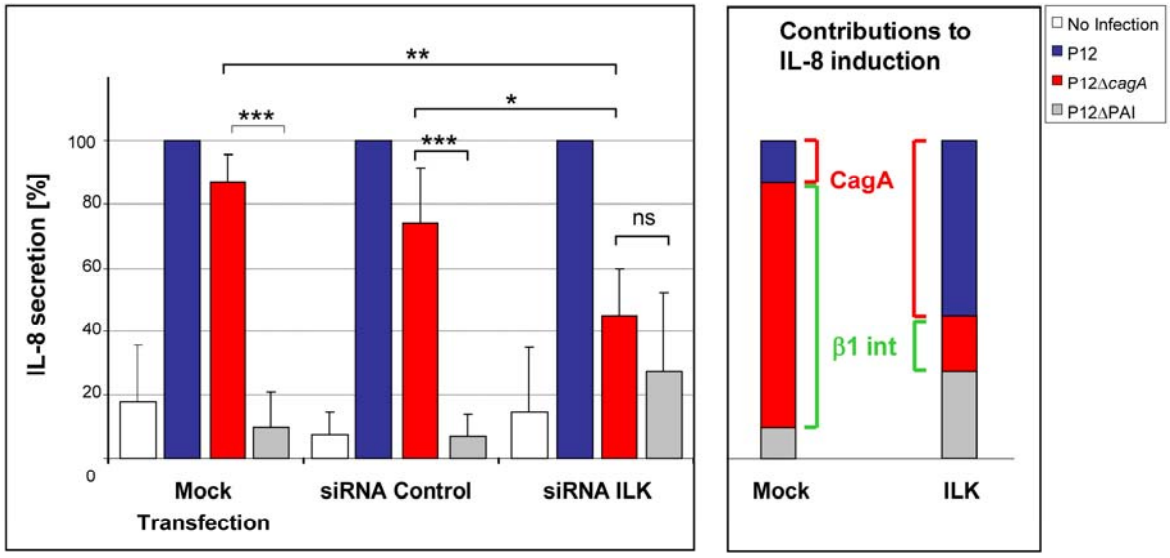


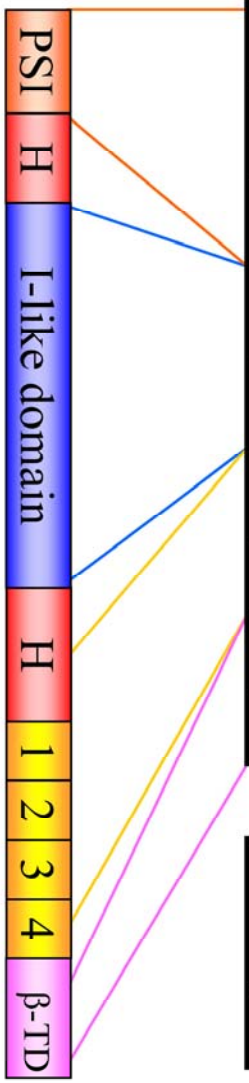
Figure 3-28 Effects of ILK on IL-8 induction via Cag apparatus

IL-8 production by AGS cells during infection with P12 wild type and mutants lacking *cagA* (Δ*cagA*) and the *cagPAI* (Δ*PAI*). Percentage relative to control infection with wild type strain for each treatment. siRNA control transfected siRNA with a non-sense sequence with similar GC content as the ILK siRNA sequence. Statistical analysis using t Test. * P<0.05, ** P<0,01, ***P< 0,005. ns, no significance.

3.3.5 Effects of integrin β1 antibodies in IL-8 induction via Cag apparatus

As the integrin β1 plays a role in CagA translocation, the IL-8 induction levels via Cag were evaluated in infection conditions were cells were exposed to antibodies against the different domains of the integrin β1 subunit. In the case of IL-8, the effect is summarized in figure 3-28. Only two antibodies showed an effect on IL-8 induction. The AIIB2 increased the IL-8 induction up to five times as compared to the control infection. On the other hand, antibody LM534 not only reduced the IL-8 induction via Cag, but also could induce up to three times the normal amount of IL-8 produced by the cells. It is worth to mention that antibodies K20, 9EG7, B3B11 were able to induce IL-8 *per se* although not in such magnitude as antibody clone LM534 (data not shown).

It seems that not only the binding of the integrin antibodies can alter the CagA translocation, as it was the case with 9EG7, but the binding of the LM534 antibody reduced the IL-8 induction by the Cag apparatus, even though it proved not to affect the CagA translocation.



Clone	Epitope	Antibody activity	IL-8
N29	14-55	Stimulatory (**)	++++
8E3	ND	Stimulatory	++++
JB1A	82-87	Inhibitory	++++
AIIB2	189-304	Inhibitory	+++++++
12G10	Conform.	Stimulatory/Inhibitory	++++
K20	426-587	Neutral	+++
9EG7	495-602	Stimulatory/Inhibitory	+++
B3B11	660-668	Stimulatory	+++
LM534	587-708	Neutral	+
3S3	Unknown	Inhibitory	++++
6S6	Unknown	Inhibitory	++++
B44	Conform	Stimulatory	++++

Figure 3-29 Effects of integrin antibodies on IL-8 induction by the Cag apparatus

Summary of IL-8 induction via CagPAI apparatus by AGS cells pre-treated with antibodies against integrin β1. Antibodies' names and linear epitopes are described here for each clone. *Conform*, for those antibodies recognizing epitopes known to be available under certain activation status of the integrin heterodimer. *Neutral* defined as not observed any effect on binding capacity of the integrins. *Inhibitory* defines antibodies that diminish the binding capacity of the integrin heterodimers. *Stimulatory* are antibodies that enhance the binding to their substrate. (++++) defines the normal IL-8 induction during infection with P12 wild type of AGS during 3 hours. The data is representative for two independent experiments. 30 µg of antibody was used on synchronized AGS cells.

3.4 Cellular processes involved with the functionality of the Cag apparatus

3.4.1 Evaluation of CagA translocation and phosphorylation

In order to elucidate the mechanisms involved in the CagA translocation into the eukaryotic cells, cellular processes that involved directly or indirectly integrin β1 signaling pathways were manipulated or disrupted using inhibitors. The effects of this inhibitors were analyzed in relation with the capacity of the Cag T4SS to translocate CagA (by CagA phosphorylation) and induce IL-8. AGS cells were used as standard cell line because of their optimal CagA-P and their known IL-8 dynamics. All results are summarized in table 3-1. Each inhibitor was initially tested with 30 minutes pre-incubation and a four-hour infection. If after these conditions a change in the levels of CagA phosphorylation was detected, experiments with different pre-incubation times (minimum 30 minutes and maximum 60 minutes) and one, two or three hour infections were repeated. The levels of CagA phosphorylation were detected through Western blot detecting tyrosine-phosphorylated proteins using the antibodies PY99 and 4G10. The CagA-P levels were analyzed qualitatively in comparison with the AGS control infection. In the case of IL-8, the induction took place for three hours. IL-8 levels were measured and analyzed taking as control the IL-8 levels induced by the inhibitors alone.

3.4.1.1 Membrane composition, proteins integrity, signaling and clustering

The first interaction of the Cag apparatus with the host cells takes place with the cell membrane and its components. To learn more about this interaction, normal processes associated with the cell membrane's function were disrupted by altering the lipid composition, by changing the protein topography of the cell membrane's surface or by disrupting some of the first signaling molecules on the cytoplasmic part of the cells. The effects of these changes on the CagA translocation and IL-8 induction were analyzed to better understand the translocation process.

3.4.1.1.1 Effects on CagA phosphorylation

In order to disrupt several membrane processes that could have an indirect effect on the function of the integrin, normal lipid composition was disrupted by the use of Methyl-β-

Cyclodextrin (MBC). MBC is reported to have high affinity for cholesterol, sequestering it from the membrane of the cells and, thus, disrupting microdomains present in the membrane known as lipid rafts (Rodal et al., 1999). Cells were pre-incubated in presence of MBC for one hour and then infected for four hours. CagA phosphorylation was strongly diminished after the depletion of cholesterol, as seen in figure 3-30A. This effect is stronger in infections with *H. pylori* strain 26695 and weaker on *H. pylori* strain P217 (data not shown). However, the effect is present on all these three strains tested.

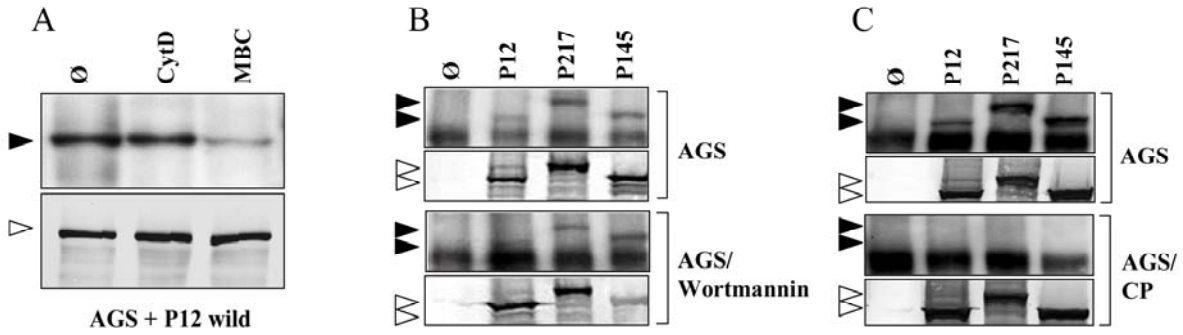


Figure 3-30 Effects of inhibitors on CagA phosphorylation

Immunodetection of phosphorylated CagA (CagA-P). Full head arrow shows phosphorylated form of CagA detected using anti-phosphotyrosine antibody PY99. Empty arrow head shows bands corresponding to CagA detected using anti-CagA AK257. (A) Effect of Cytochalasin D (CytD, 1 µg/ml)) and cholesterol depletion by Methyl-β-Cyclodextrin (MBC, 0,1 g/ml). (B) Effect of Wortmannin (10 nM) and (C) Calpeptin (CP, 280 µM). Infection time 3 hours with MOI 60

Another aspect evaluated was the protein composition on the surface of the cells by altering it through a pre-digestion using trypsin (10 µg/1x10^6 cells) and thrombin (4 U/1x10^6 cells). These two proteases are known to digest some proteins on the surface of the cell without entering into the cytoplasm. In both cases, the changes of the membrane topology by the pre-digestion of membrane proteins on the cells did not change the levels of CagA phosphorylation measured by immunoblot (data summarized in table 3-1).

In the membrane signaling, PI3-K and PLC are considered important signaling molecules that, through their kinase and phospholipase activity, transport signals from the membrane into de interior of the cell. Their function is reported to be disrupted by Wortmannin (PI3-K) and U73122 (PLC). In both cases, no changes on CagA phosphorylation were observed (see figure 3-30B and table 3-1).

By disrupting in several ways the membrane function (composition of the proteins on the membrane surface, lipid composition and intracellular signaling) it was discovered that only a massive depletion of cholesterol had an effect on CagA-P, being directly correlated with the

exposure time of the cells to MBC. Since lipid rafts have been associated with the capability of clustering of the integrins (McDowall et al., 2003), it is possible that the inhibition of such clustering damages the translocation capability of CagA. To confirm this hypothesis, the effect of calpeptin, a Calpain inhibitor that reportedly causes inhibition of clustering of the integrins, was studied. In accordance with the results obtained by disruption of lipid rafts function, the use of calpeptin inhibited as well the CagA-P (Figure 3-30C).

3.4.1.1.2 Relevance of membrane processes on IL-8 induction

By disturbing lipid rafts with the use of Methyl-β-Cyclodextrin (MβC), CagA-P was reduced. However, in the case of IL-8, only in infections with *H. pylori* strain 26695 was the IL-8 induction strongly reduced. In infections with P12 strain, the IL-8 induction increased almost 15% in a statistical significant way (Student t Test P=0,02) as seen in figure 3-31.

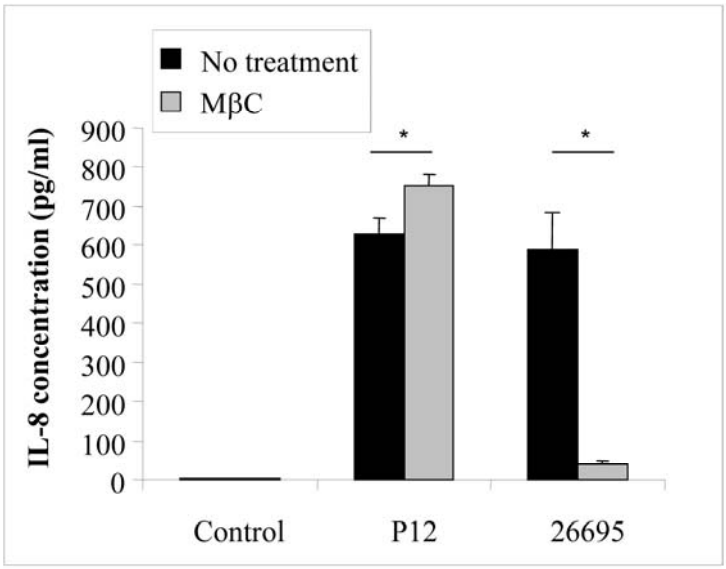


Figure 3-31 Effect of Cholesterol depletion on IL-8 induction via Cag apparatus

Depletion of cholesterol achieved after 1-hour pre-treatment of AGS cells with Methyl-β-Cyclodextrin 0,1 g/ml. IL-8 concentration in pg/ml after a 3-hour infection of AGS cells with strains P12 and 26695, MOI 60. Statistical analysis using t Test. * P<0.05

By pre-treating cells with trypsin and thrombin, some of the extracellular proteins were digested. At the same time, trypsin in PBS causes the detachement of the cells. To analyze the effect of the detachment, cells were pre-incubated in media:PBS solution in a 1:3 proportion and added trypsin 10 µg/ml. This treatment does not cause detachment of cells but conserves the trypsin activity. By analyzing the IL-8 induction, the induction levels were higher in cells treated with trypsin compared with the control cells (see figure 3-32). However, there is also a

statistically significant increment of IL-8 induction if cells are detached from the substrate by the trypsin.

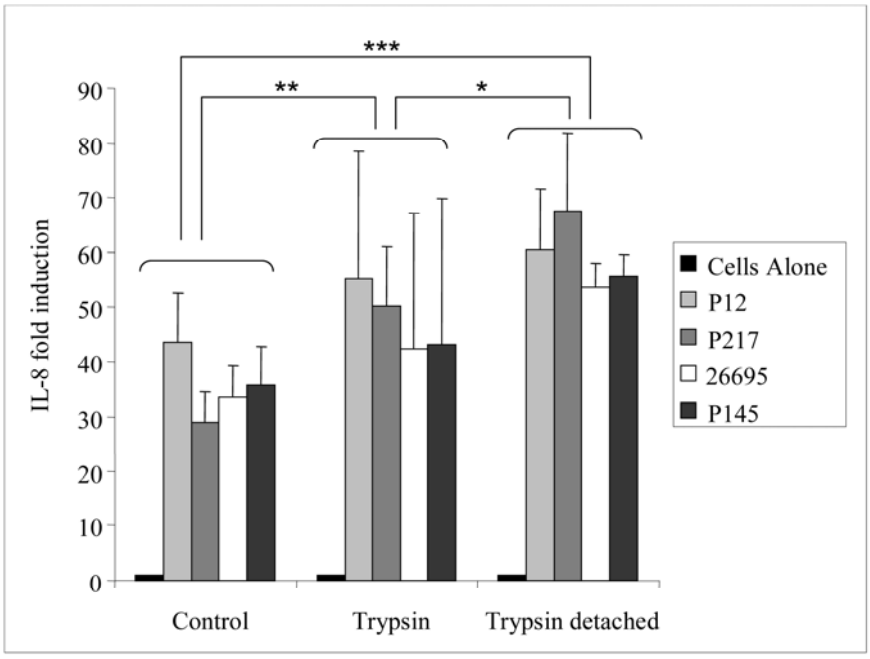


Figure 3-32 Effect of trypsin pretreatment of AGS cells in IL-8 induction via Cag apparatus

IL-8 fold induction relative to –pretreated AGS cells not infected. trypsin (20 μg/ml) exposure of cells 30 minutes before a 4-hour infection with wild type strains P12, P217, 26659 and P145. Statistical analysis using t Test. * P<0.05, ** P<0,01, ***P< 0,005.

3.4.1.2 Endocytosis involvement in Cag functionality

Until today, the process by which the Cag apparatus can translocate CagA into the cell's cytoplasm is not well understood. Since most of the molecules enter the eukaryotic cell by an endocytic process, the effect of disturbing the endocytic system was evaluated on CagA translocation as well as on IL-8 induction.

3.4.1.2.1 CagA phosphorylation requires some endocytic components

Integrin β1 has been linked with endocytic processes, as it has been described for the interaction between invasin (Inv) protein and integrin $\beta_1\alpha_5$. The effects on CagA translocation by the disruption of cytoskeleton using Cytochalasin D, Latrunculin A and Jasplakinoline, as well as by the disruption of the microtubule system by Nocodazole, were evaluated. Cells were pre-treated with these inhibitors for 30 minutes before infection followed for one, three or four hours with an MOI 60. No effect was observed in the CagA-P levels with the inhibitors in comparison to control cells. This shows that the cytoskeleton and microtubule

system are no relevant cellular processes for the CagA translocation/phosphorylation (data summarized in table 3-1).

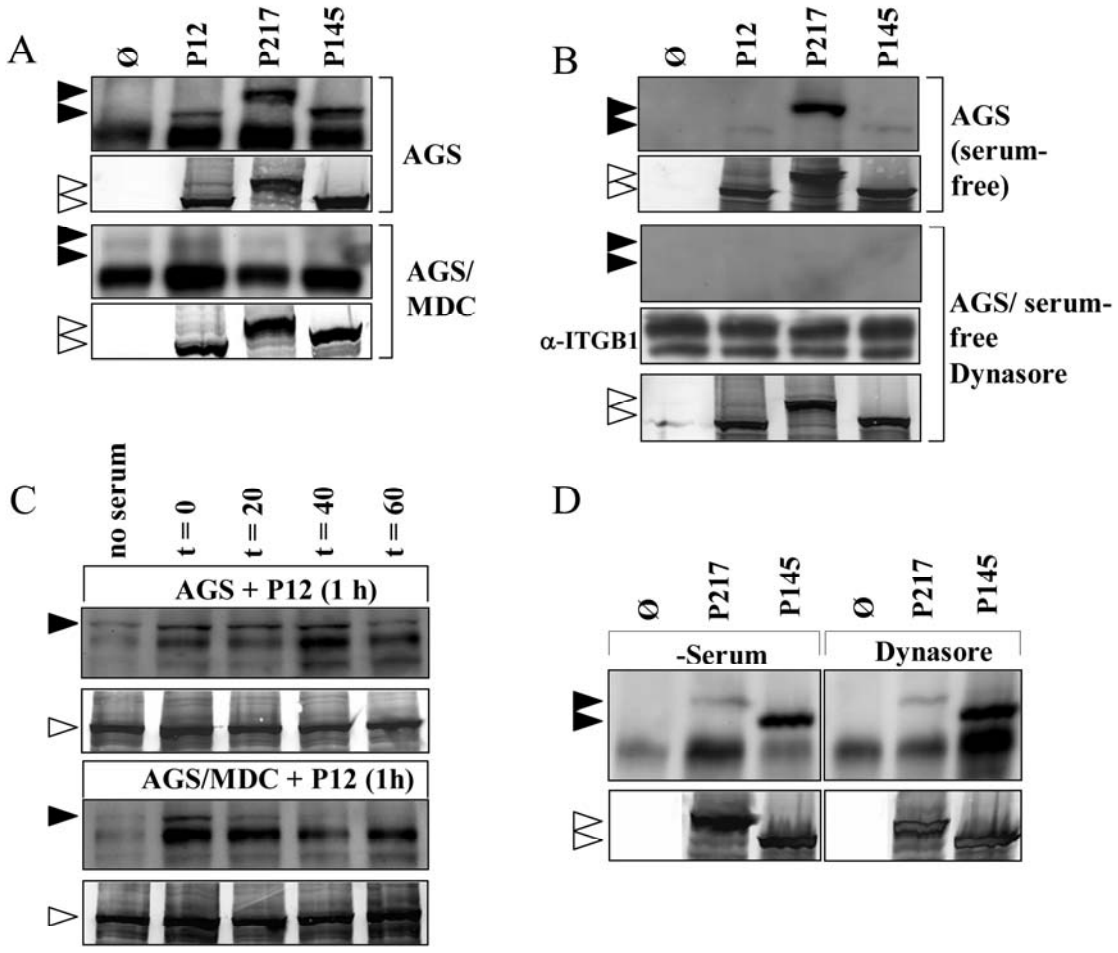


Figure 3-33 Clathrin and CagA phosphorylation

Immunodetection by western blots of phosphorylated CagA after infection of AGS cells pretreated with Mono-dansylcadaverine (MDC, 0,3 mM) or Dynasore (20μM. Full head arrow shows phosphorylated form - detected using anti-phosphotyrosine antibody PY99and 4G10. Empty arrow head shows bands corresponding to CagA detected using anti-CagA antibody AK257. (A) and (B) show CagA-P levels after 30 minutes pretreatment of AGS cells with inhibitors and 1-hour infection MOI60. α-ITGB1, detection of integrin β1 using clone LM534 in no reducing conditions. (C) and (D) show data testing effect of inhibitors on bacteria

Clathrin is a protein involved in endocytosis processes and until today, there are controversial reports of its association with the cytoskeleton (Cupers et al., 1998; Kirchhausen, 1999; Mousavi et al., 2004; Owen et al., 2004; Puertollano, 2004; Rappoport et al., 2006; Wu et al., 2001; Yarar et al., 2005). Mono-dansylcadaverine (MDC) and Dynasore are specific inhibitors of the clathrin pathway. The first one seems to inhibit the recycling of the clathrin chains by stabilizing nascent clathrin-coated vesicles (CCV) and preventing the uncoating of

already formed CCVs (Phonphok and Rosenthal, 1991). Dynasore has been described to inhibit directly the ATPase dynamin, which is required in different steps of the formation of clathrin-coated pits (CCP) and CCV (Macia et al., 2006; Nankoe and Sever, 2006). In the case of MDC, a 30 minutes pre-treatment of cells with 0,3 mM concentration, was able to abolish the CagA translocation and phosphorylation, both for four hour experiments as well as for one hour (Figure 3-33A).

The effect of dynasore was evaluated with *H. pylori* strains grown on serum-free plates for at least two passages to remove all serum present on them, because of dynasore sensitivity to serum proteins. Shortly before infection, cells were washed three times with PBS (-Ca^{+2}, -Mg^{+2}) and once with serum-free RPMI. Then in serum-free media, 80 μM dynasore was given 30 minutes before a one hour infection with bacteria previously resuspended in serum-free media. The same effect seen with MDC was present with dynasore. CagA phosphorylation was completely abolished (Figure 3-33B).

The effect of both compounds on *H. pylori* were unknown. Therefore, their effect on the functionality of the Cag apparatus was tested in time experiments. In the case of MDC, AGS cells were synchronized and exposed to the MDC from time point 0. Then, cells were infected for one hour at the time points 20, 40, 60 and 80 minutes. The experimental setup garanties that all bacteria are exposed only one hour to the chemical and any effect seen would be associated to an effect of the inhibitor on the clathrin pathways in the cell. As seen in figure 3-33C, there are diminished levels of CagA-P in a time dependent manner, indicating that MDC is not disturbing the Cag apparatus, but its way of action is localized on the eukaryotic cell's side.

To examine the effect of dynasore on the bacteria, a time experiment was not possible since dynasore enters quickly into the eukaryotic cells and its effects are almost immediately seen. However, to evaluate its effects on the bacteria, the bacteria suspension was exposed directly to 80 μM concentrations of dynasore for 45 minutes and washed two times to eliminate any excess. These bacteria were used to infect AGS cells for one hour in CM at 37°C with 5% CO_2. The translocation was not affected (Figure 3-33D), indicating a non toxic effect of the dynasore on the *H. pylori* ability to translocate CagA during the infection.

In view of the fact that proper function of the clathrin pathway is clearly necessary for the CagA translocation, one can assume that clathrin-dependent endocytic processes are essential for it. If this is the case, it is necessesary to explain the way CagA escapes the lumen of the endosome into the cytoplasm to be exposed to the Src kinase to be phosphorylated. Taking as

example virus and hormones, who seem to have discovered how to be released from the endocytic lumen into the cytoplasm, experiments were designed to disturb these mechanisms. They involve acidification of endosomal compartments in the case of viruses; or changes in the membrane potential of the endosome with or without involvement of chaperone proteins as Hsp90, as it was described for FGF-1 (Fibroblast Growth Factor -1) (Wesche J, 2006). For each of these processes, inhibitors were used. To inhibit acidification, the inhibitors Bafilomycin A, Concanamycin A and ammonium chloride (NH_4Cl) were tested. Monensin A disrupts the membrane potential and Geldanamycin inhibits the Hsp90. Their effects were evaluated with a pre-incubation of cells for 30 and 60 minutes followed by a one-hour infection. As shown in Table 3-1, none of them had an effect on the CagA-P levels in comparison to control cells (immunoblots in figure 3-34). However, these results cannot rule out a mechanism for the apparatus that requires the formation of clathrin coated vesicles and a system to escape from their lumen into the cytoplasm. Further studies are required in order to answer this important question.

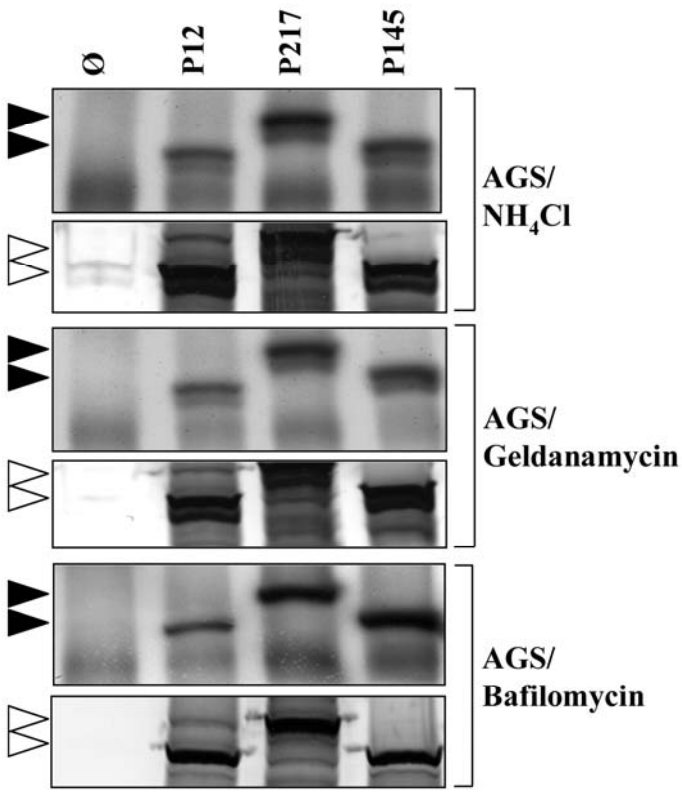


Figure 3-34 Involvement of endocytosis processes in CagA phosphorylation

Immunodetection of phosphorylated CagA. Full head arrow shows phosphorylated form detected using PY99 antibody. Empty arrowhead shows bands corresponding to CagA detected using AK257. Effect of ammonium chloride (NH_4Cl, 20 mM), Geldanamycin (50 nM) and Bafilomycin 1A (1 ng/ml). Infection time: one hour. MOI 60 using wild type strains P12, P217 and P145.

3.4.1.2.2 Involvement of endocytic processes in IL-8 induction

In the case of endocytosis processes, all inhibitors tested for CagA phosphorylation were also tested in their effect on Cag dependent IL-8 induction. Some of the data are shown in figure 3-35 and all effects are summarized in table 3-1. This data has to be interpreted carefully since the inhibitors can as well disturb the production of IL-8 and/or is released into the media. There is only a drastic inhibition of IL-8 Cag-dependent induction in pre-treatments with Mono-dansylcadaverine (MDC), and a less but also significant reduction with ammonium chloride (NH_4Cl), Concanamycin A, Monensin A and Bafilomycin A. No significant changes are observed with Nocodazole or Wortmannin.

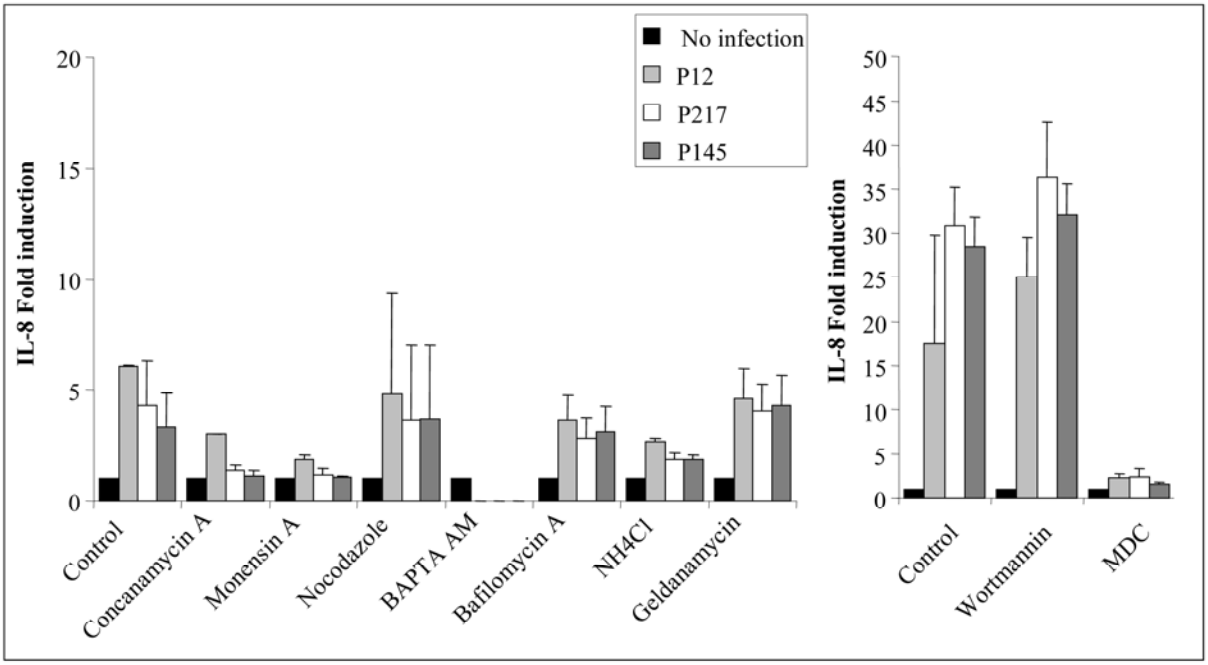


Figure 3-35 Effect of endosome processes inhibitors on IL-8 induction via Cag apparatus

IL-8 fold induction relative to pre-treated AGS cells not infected. IL-8 induction by wild type strains P12, P217 and P145 on pre-treated cells after 3 hours infection MOI 60. Control, AGS cells with no inhibitor, infected. MDC, Mono-dansylcadaverine. Data correspond to at least two independent experiments.

3.4.1.2.3 Cag apparatus and clathrin

As already observed using inhibitors for clathrin and dynamin, clathrin seems to play an important role in the function of the Cag apparatus. To confirm this visually, immunofluorescence was done where co localization of GFP wild type bacteria and immunodetected clathrin was revealed (see figure 3-36A). Given the fact that only 5-9% of bacteria present the Cag apparatus at the infection time, we tried to confirm that it is a Cag dependent event. The apparatus was detected using antibodies against the CagY RRII and

looked for co-localization with clathrin. As exposed in figure 3-36B, there was co-localization of CagY with clathrin.

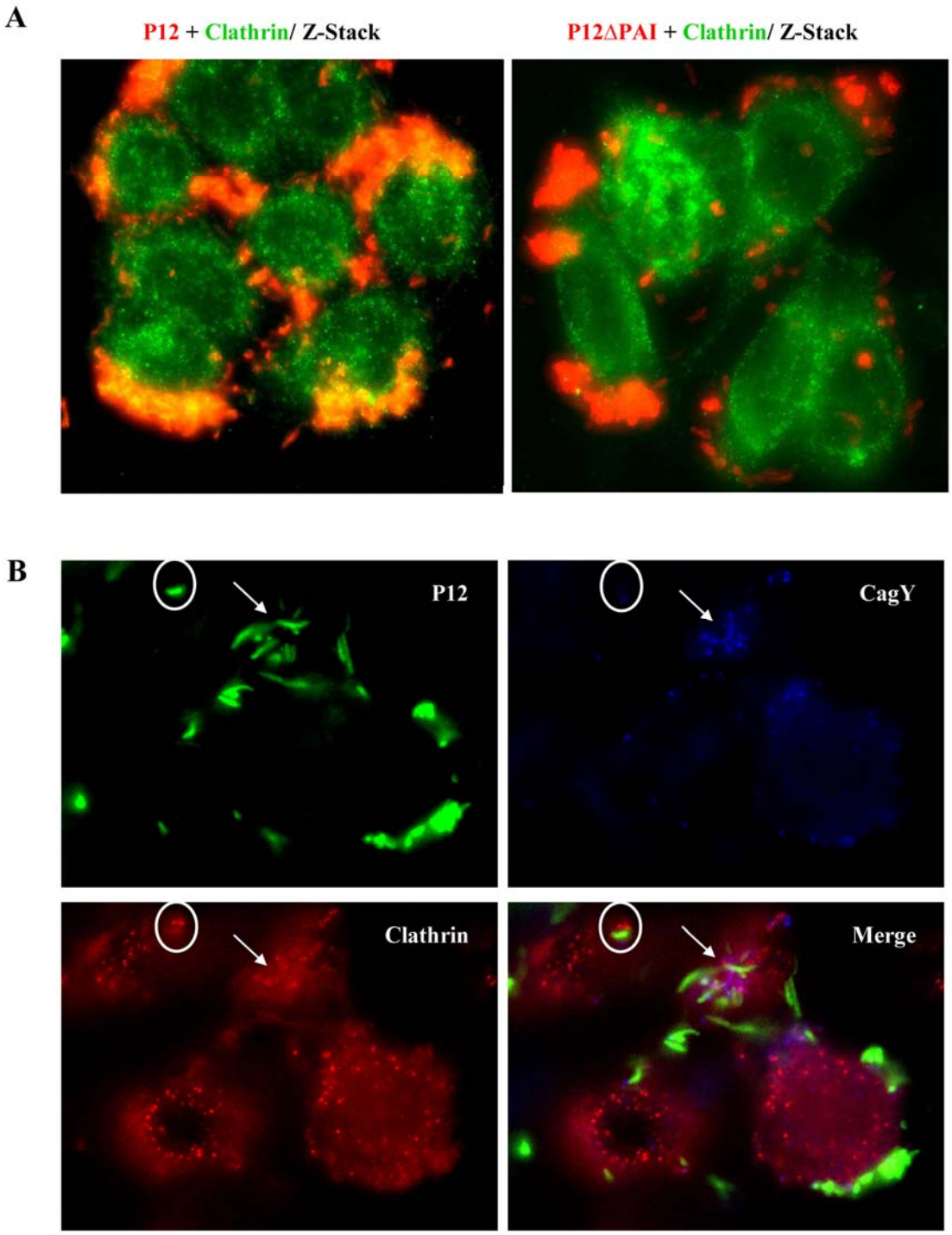


Figure 3-36 Co-localizations of CagPAI containing bacteria and its CagY with clathrin

(A) AGS infected with GFP-expressing P12 wild type strains or P12ΔPAI for one hour were fixed and permeabilized in order to immunodetect clathrin heavy chain (red). Z-stack sections were taken with 0,2µM and merged to one slide. Only P12 wild type co localizes with clathrin molecules. (B) P12 GFP expressing bacteria (green) after 30 minutes infection of AGS cells were fixed and CagY as marker for apparatus (blue) was immunodetected using a $Alexa_{647}$-conjugated antibody (AK273) and clathrin heavy chain using a secondary antibody $Alexa_{555}$ conjugated (Red). Arrows point at co-localization places of CagY and clathrin

3.5 EM studies of the Cag apparatus

3.5.1 Structure of the Cag apparatus

The structure of a Type IV Secretion apparatus is unknown until today. In collaboration with Dr. Valério Matias, from the Max Planck Institute for Biochemistry, in Martinsried, Germany; aproject was started with the purpose to elucidate the structure of the Cag apparatus using Cryo Electron Microscopy Tomography (Cryo-EM). The first step was to solve a problem noticed in previous Electron Microscopy (EM) studies: the low amount of Cag apparatus on the surface of *H. pylori*. Therefore, the first effort was to discover a stimulus for the formation of the apparatus.

During previous experiments, different conditions were tested to obtain the best CagA translocation and phosphorylation in AGS cells. It was observed that infections with strains P12 and 26695 of AGS cells in PBS solutions without divalent cations, reduced by a factor of four the time normally necessary to observe a strong CagA-translocation/phosphorylation related phenotype called the "Humming bird" phenotype. At the same time, it was observed changes in the consistence of the PBS after long exposure of the cells to it. What originally was a fluid solution, changed to become visible viscous, without changes in turbidity.

With these observations, a new protocol was designed to stimulate the bacteria to produce the Cag apparatus without the direct contact of eukaryotic cells. AGS cells were seeded and left 24 hours to grow in normal conditions to a confluency of 70% to 80%. Cells were washed and exposed to PBS free of calcium and magnesium for 60 minutes in low CO_2 conditions at 37°C. The PBS was collected, sterile filtrated and named AGS exudate. Bacteria were resuspended in this exudate and incubated for 90 minutes in 5% CO_2 at 37°C; afterwards they were prepared with negative staining for the EM.

About 20% to 30% more pilus structures were observed in strains 26695 when exposed to the AGS exudate in comparison to the ones in PBS control solution. The structures observed are depicted in figure 3-37. Two forms were identified, a "naked" form and a "sheathed" form. The naked form has a diameter of 20-25 nm, and seems to be formed by regular subunits arranged in a helical macrostructure that contains an inside channel of 2-3 nm diameter. The sheathed form contains the "naked" form covered by a 10 nm dick "coat", which has not been identified yet. The sheathed structures appear to be attached to bacteria cells as seen in figure 3-38.

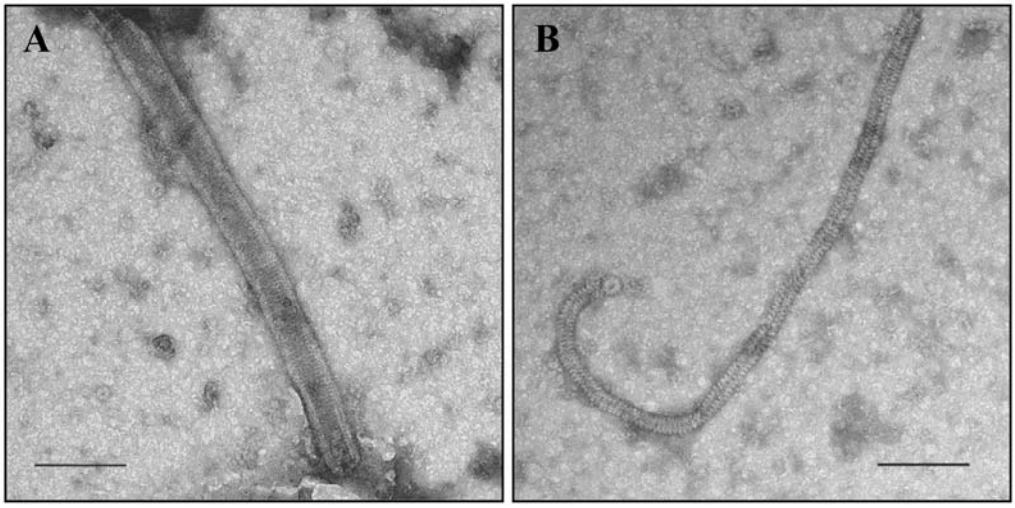


Figure 3-37 Cag apparatus sheathed and naked forms

Electron microscopy pictures of the (A)“sheathed” form and the (B)”naked” form pilus like extensions after 90 minutes stimuli of strain 26695 with AGS exudates. Preparation as negative staining. Bars indicate 50 nm.

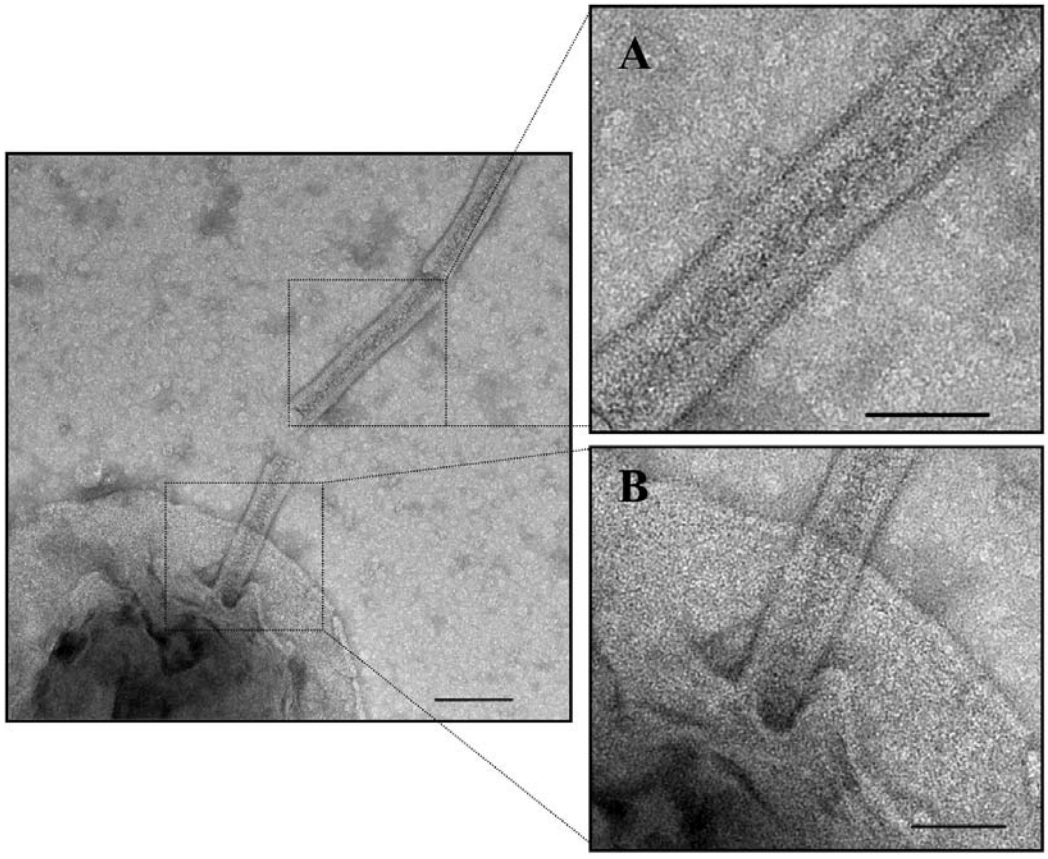


Figure 3-38 Cag apparatus attached to bacteria

Electron microscopy pictures of the (A)“sheathed” pilus like structure and the (B) connection part with the bacterium formed after 90 minutes stimuli of strain 26695 with AGS exudates. Preparation with negative staining. Scale bars represent 50 nm

3.5.2 CagY is not part of the sheath, but CagC constitutes part of the naked form

It was observed that the mutant 26695Δ*cagY* did not present any sheathed form, but only the naked one. Because of this observation, the first evaluation was to prove that the CagY was found as part of the sheath. Using the antibody AK273 that recognizes the CagY repeat region II (AK273), immunostaining was done from purified "pilus-like structures" from *H.pylori* strain 26695. The immunolabeling did not stain the sheathed or the naked structure (data not shown).

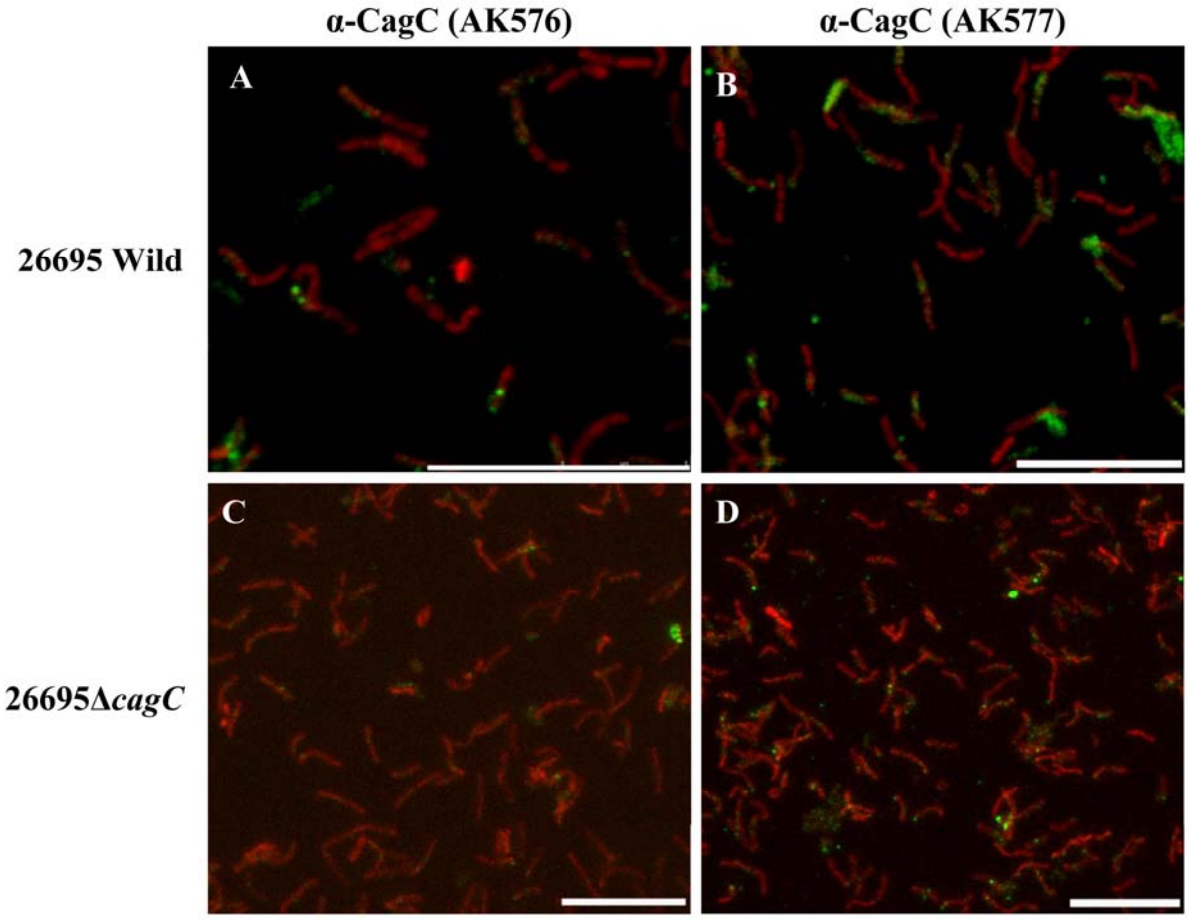


Figure 3-39 Specificity of antibody against CagC in immunofluorescence

Strain 26695 and the *cagC* mutant (26695Δ*cagC*) are stained using DAPI (red) to stain their DNA. Antibodies against CagC, AK576 and AK577, were added in concentration 1:1000 to 1% PFA fixated bacteria and labeled with an anti-rabbit secondary antibody $Alexa_{488}$ labeled in dilution 1:2500 (green). Bars represent 10μm.

It was tried to identify the protein that composes the naked form. Based on its regularity and on the homologies to the VirB system, it was postulated that this protein could be CagC. For the detection of CagC protein two antibodies were developed, the AK576 and the AK577. In

immunostaining experiments, only the AK576 stained the bacteria in a specific way (Figure 3-39). The anti-CagC antibody AK576 shows a high specific binding, with very low background staining in the *cagC* mutant, contrary to the case of AK577 antibody, which stains bacteria in an unspecific manner (Figure 3-39D).

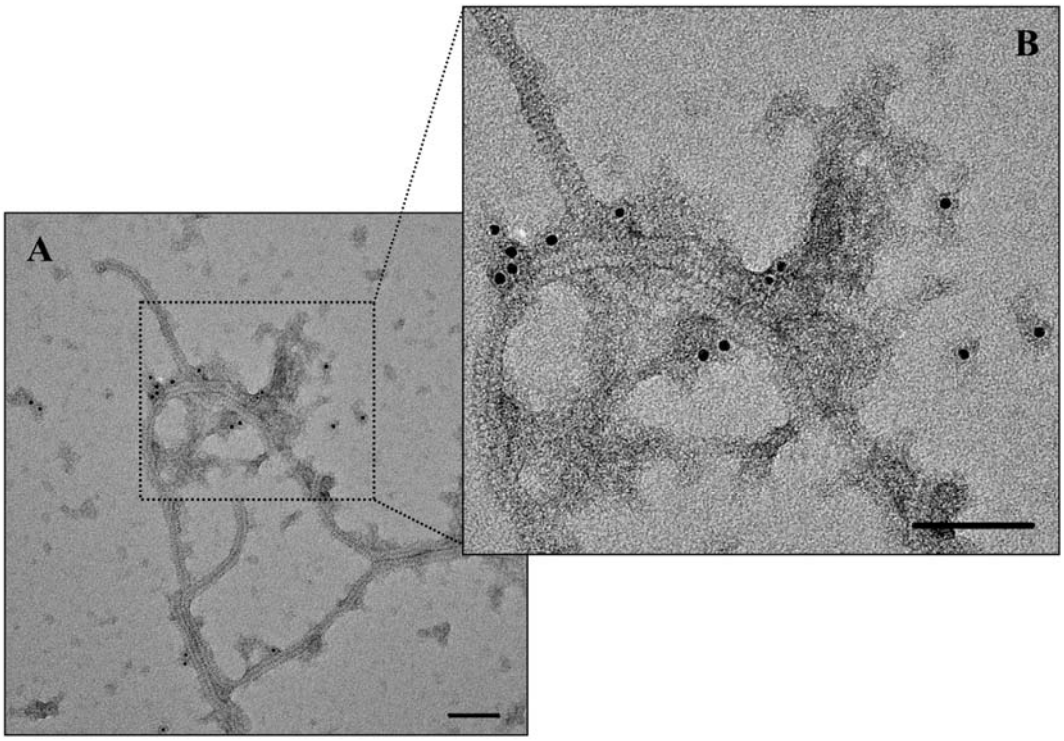


Figure 3-40 EM Immunogold detection of CagC on the apparatus

After induction of formation of the pilus-like structures, the "naked" form was immunostained using an anti-CagC antibody (AK576) as primary antibody and an anti-rabbit IgG gold labeled secondary antibody. Bars represent 100 nm.

Since anti-CagC antibody AK576 is specific for CagC, 26695 strains were induced in AGS exudate as done before. Their pilus-like extensions were gold immunostained for EM pictures. As seen in figure 3-40, gold particles associate with the "naked" form seen before. The gold particles associate only with those subunits that have broken apart from the pilus-like structure, and they are found in the part that forms the inner channel observed in this sheathed form. This data confirms that CagC protein is a component of the "naked" form of the apparatus.

4 Discussion

4.1 Cag apparatus effect on cell migration

The motility of cells, especially of hematopoietic cells, is essential for the correct function of the immune system. In the last years, there have been great progresses made in the understanding of the mechanisms that allow immune cells to respond to an infection, move towards a tissue and find their target. Molecules like fMLP or IL-8 are sensed by leucocytes, inducing a cascade that ends in the polarization of the cells. This polarization is essential for movement towards the stimulant's source. The motility towards the attractants source is based on a sense of a concentration gradient (Futrelle, 1982). This allows the cells to find the place where they are needed. To be able to investigate an effect on the motility via the Cag apparatus, the motility of the cells through a membrane and towards a chemoattractant using a Boyden Chamber was tested. In these experiments was observed that independently from the chemoattractant, cells are disturbed in their capacity to cross the membrane in a CagA dependent manner (Figure 3-2). Even though there is a reduction of about 60% motility, it is not completely lost. These results left the question open in which part of the motility associated processes, the CagA was acting. To answer this question, imaging studies were necessary.

As part of the physiological function of immune cells, leucocytes travelling in the circulatory system attach to the endothelial cells forming the blood vessels as a response to chemoattractants. They achieve this through interaction with selectins and the involvement of integrins. Once cells have attached, they polarized by forming a migration front or lamellipodia, and a migration rear or uropod (Zigmond, 1978). The formation of these two parts is necessary to start migrating towards the tissue between or through the endothelial cells (diapedesis). In this step, a movement on the surface of the endothelial cells' membrane is required, and integrins have been described to play an essential role in it (Lock et al., 2008). Because the movement takes place over a surface, it could be considered as a 2D movement. This kind of movement was observed in the experiments using the agarose assay. In the "2D" agarose system, cells are able to form a lamellipodia and a uropod, presenting the polarization essential for motility (Figure 3-3). After infection with *H. pylori* strains expressing CagA, cells are still able to migrate, however in an erratic manner. They presented modified and bizarre forms of the uropod as well. This leads to the question, which of all effects related to

CagA phosphorylation is responsible for this? Phosphorylated CagA has been reported to disturb the function of FAK and, in this way, the connection of integrins with the cytoskeleton (Tsutsumi et al., 2006). If that is the case in our experimental setup, no lamellipodia could be formed. Lamellipodia are formed by assembly of Focal Adhesions, which support the traction of the cytoskeleton and move the cell forwards (Broussard et al., 2008). The fact that lamellipodia are still observed indicates that CagA is, in this case, not acting on the function of FAK. This could be the consequence of the very low MOI used in these experiments (MOI 5) and the fact that CagA is not effectively translocated into these cells.

What happens with leucocytes on their way into the tissue? It was assumed that integrins were responsible for the motility of the cells once they had crossed the endothelial barrier and they were in the interstitial tissue. Their function was to interact with the Extracellular matrix (ECM) proteins and facilitate the movement. This idea was until lately reformulated (Lammermann et al., 2008). Lammermann et al. have demonstrated that cells, once they are immersed in the interstitial compartment, need to turn off the activity of the integrins in order to move through it. If integrins are activated as the cells move inside the tissue, they stop the motility and lose the polarization. Considering this observations, a different point of view could be used to analyze the effect that *H. pylori* has on the behavior of dHL-60 cells migrating in a 3D collagen matrix. As seen in figure 3-4, all cells infected with wild type strain, lost any sign of polarization. It was not the case for infections of dHL-60 cells with the *cagA* mutant, where some cells are still able to polarize in the collagen matrix. For cells infected with *H. pylori* lacking the *cagPAI,* there was no difference at all with the uninfected cells. Based on this, it is possible that CagA is able to activate the integrins and, by doing so, inhibits the movement of cells into a three dimensional system.

CagA reduces the motility of dHL-60, which are used as example for leucocytes movement (Figure 3-4). Future research should answer if this occurs as well in primary leucocytes, and if the effect on 3D motility is caused by activation of integrins in a direct or indirect way. There is the possibility that activation could be already caused from outside, since CagA translocation is reduced in these cells. It would make the CagA a very good immunomodulator as extracellular toxin if bacteria were able to secrete some of it in the environment. In this way, the toxin could interfere with the immune cells function without the need of physical contact between bacteria and cells. There are several bacterial proteins able to manipulate at least one of the two main systems associated with motility, as they are the cytoskeleton and the GTPases Rho, Rac and Cdc42. Some of the cytoskeleton disrupting bacterial proteins are ActA from *Listeria monocytogenes*, IcsA from *Shigella flexneri* and Tir

from *E. coli* EPEC. These proteins modify the cytoskeleton by manipulating the actin polymerization through changes that lead to modified Arp2/3 complex function (Ahmadian et al., 2002). On the GTPases, bacterial proteins can act as functional mimicry taking over the function of the eukaryotic GAPs (GTPase Activating Proteins) and GEFs (Guanine-nucleotide Exchange Factors). These proteins are responsible for controlling the transition state of the GTPases. By controlling their transition state, proteins like YopE (*Yersinia spp.*), ExoS (*Pseudomonas aeruginosa*), SptP and SopE / SopE2 (S*almonella typhimurium*) are able to alter the host phagocytic process and invade the host cells or avoid phagocytosis (Galan, 2001; Heesemann and Aepfelbacher, 2001; Zhou et al., 2001). At the same time, the function of the GTPases can be modulated by covalent modifications (Aktories and Barbieri, 2005) as caused by the toxins ADP-ribosyltransferase C3 from *Clostridium botulinum* (Wilde et al., 2000) and *E. coli* cytotoxic necrotising Factor 1 (CNF1) (Fiorentini et al., 1997). Although many bacteria pursue the objective of getting inside the cells to survive in them, *H. pylori* remains mostly extracellular. *H. pylori*'s Cag T4SS has been associated with changes in the cytoskeleton and CagA has not been found to interefere with any of these ways until now (Al-Ghoul et al., 2004), making a possible extracellular effect on motility a new mechanism for a bacterial protein to disturb the motility of cells.

4.2 Integrin β1 as receptor of the Cag apparatus

4.2.1 Integrin β1 is necessary for CagA translocation

Part of the studies with dHL-60 cells revealed a difference in the CagA translocation in comparison to their parental cell line HL-60 (Figure 3-5). The comparison of their mRNA and proteome indicated the possibility that integrins could play an important role in CagA translocation. This necessity was further demonstrated as the integrin β1 subunit was found to be essential for CagA translocation (Figure 3-6). To better understand the role of integrin in the CagA translocation, it is important to know more about the integrin family.

Integrins are a family of heterodimeric transmembrane proteins which to date include more than 28 recognized members. They are composed of an alpha (α) subunit in combination with a beta (β) subunit. One subunit cannot work without the other, and because of this property, several of the functional mutations used for their study concentrate in only one of the subunits. As members of the transmembrane protein category, integrins are composed of a cytoplasmic tail (20-60 amino acids), a short transmembrane domain and an extracellular

domain (700-900 amino acids) (Travis et al., 2003). Each domain possesses a function, which has been carefully studied over the last 25 years. The first member of the family to be discovered was the integrin β1, in that time called fibronectin receptor and today denominated CD29 or ITGB1. This protein showed an amazing capacity to resist trypsin digestion (Sczekan and Juliano, 1990). Their study gave a better understanding of the importance of the extracellular matrix (ECM) milieu in the physiology and function of cells. With the discovery of the interaction of ECM proteins with integrins, their remarkable property of not only transmitting signals from the outside of the cell to the inside, but as well to respond from the inside environment of the cells with changes on the outside was found. They achieve this by modifying their affinity capacity to ligands. This behavior has been called inside-out signaling. In this way they can regulate adhesion of the cells to their surroundings, initiate and enhance signaling cues from growth factors and mediate processes linked to the cytoskeleton (Pozzi and Zent, 2003). This explains their presence in all types of cells and its essentiality in the embryonic development in the mouse (Fassler et al., 1995). Since integrin β1 is present in all cells of mammals (except on erythrocytes), it makes it an excellent molecule that will allow access to all kind of cells, independent of their origin and function. Many pathogens have discovered these advantages and exploit the integrins as triggering molecule to enter the cells. This is the case for *Yersinia spp.* through their invasin protein (InvA) (Isberg and Leong, 1990) and its direct interaction with the integrin heterodimer, or as binding surface through ECM proteins as it is the case of *Staphyloccocus aureus* (Sinha et al., 1999).

It is now possible to add *Helicobacter pylori* and its Cag T4SS to the growing list of bacteria that uses integrins. The essentiality of integrin β1 as receptor for the CagA translocation was not only confirmed but also a direct correlation was established between the amounts of integrin β1 found on the surface of the cells and the CagA translocation/phosphorylation (Figure 3-7).

4.2.2 Integrin β1 signaling and CagA translocation

As part of their inside-out signaling, integrin β1 (ITGB1) interacts via their cytoplasmic domains with proteins linked to cytoskeletal functions like Talin, Cortactin, ILK (integrin-linked kinase) and FAK (Focal Adhesion Kinase). The function of integrins has been defined as being dependent on the level of activation and their capacity of aggregation within membrane compartments or lipid rafts (Leitinger and Hogg, 2002). They have not only been shown to interact with cytoskeletal proteins, but as well with other receptors, such as the

Epithelial Growth Factor (EGF) Receptor or EGFR. (Kuwada and Li, 2000). Considering the importance of the signal transfer through the membrane, it was necessary to define its relevance in the interaction between the eukaryotic cell and the Cag apparatus. Therefore, experiments followed, which evaluated the importance of the cytoplasmic domain and one of the signaling pathways (ILK) associated with integrin β1 on CagA translocation (Figure 3-8). The fact that the expression of only transmembrane and extracellular domain of integrin β1 renders CagA-translocation non-permissible cells (CHO-K1) permissive to CagA translocation is remarkable. Together with the results showing that disruption of the ILK pathway in AGS cells does not influences the CagA translocation levels, lead to the conclusion that the interaction between Cag apparatus and integrin β1 requires neither the cytoplasmic tail nor the ILK pathway in order to translocate CagA into the eukaryotic cell effectively. This defines a novel form of interaction with integrin β1, since all pathogens described to date, which are able to interact with integrins, do so in order to exploit the integrin's capacity to relay information from the extracellular environment to the cytoplasm of the cell, starting signaling processes that would give pathogens some kind of advantage. These advantages are to facilitate invasion (*Yersinia spp.* (Hamzaoui et al., 2004), *Bordetella spp.* (el and Skurnik, 2001), *Shigella spp.* (Nhieu and Sansonetti, 1999), *Salmonella spp.* (Kingsley et al., 2004), *Streptococcus pyogenes* (Cue et al., 2000) and *Staphylococcus aureus* (Fowler et al., 2000)) or to intensify their adherence for persistence (*E. coli* (Frankel et al., 1996)). Therefore, for *H. pylori* there has to be an advantage in order to use integrin β1 as a receptor as well. The next aspects evaluated in the relation between Cag apparatus and Integrin β1 should help to elucidate this advantage.

4.2.3 The role of integrin β1 in CagA translocation is through direct interaction of Cag apparatus components and the integrin β1 subunit

Many of the above mentioned bacteria are able to interact with the integrin β1 in an indirect way. Most of them through interaction with ECM proteins as fibronectin and fibrinogen, which are able to bind through their RGD motifs to the alpha subunit of the integrin heterodimer (Krysko et al., 2004) (Smith and Cheresh, 1990). Therefore, it was necessary to determine if the Cag apparatus functionality involved a direct or indirect interaction with integrin β1. To achieve this, experiments testing the binding of soluble integrin $\beta_1\alpha_5$ (Figure

3-10 and 3-11) to *H. pylori* in a Cag dependent manner were performed. As a result, it was observed that the soluble integrin $\beta_1\alpha_5$ was able to bind to the bacteria in a Cag dependent manner. There was less background binding when bacteria were grown on serum-free conditions and, therefore they were free of ECM proteins. This correlation between Cag apparatus presence and integrin β1 was observed in infection conditions as well, where integrin β1 on AGS cells showed a co-localization with bacteria containing Cag apparatus proteins (Figure 3-12). It is interesting to see that using two different experimental setups it was confirmed what in previous EM experiments was observed (Rohde et al., 2003). Only a small percentage (5% -12%) of *H. pylori* bacteria (wild type strain) showed a functional apparatus, an apparatus able to interact with the integrin β1 (Figures 3-11C and 3-12B).

Not all these experiments can exclude the possibility that integrin interaction could take place with the alpha subunit through its I-domain, which binds to ECM proteins (Lee et al., 1995). This possibility was open since one of the proteins essential for the Cag apparatus functionality contained a RGD domain. The protein is CagL, encoded by the *hp0539* gene in *H.pylori* strain 26695. Experiments done with the invasin (Inv) protein of *Yersinia spp.* showed that by using RGD containing peptides, the binding of Inv was inhibited, possibly through competition for the RGD binding domain (Van Nhieu and Isberg, 1991). Following the same principle, the CagA translocation was tested in presence of RGD peptides (RAD peptides as control), the invasin fragments Inv397 and Inv197 (minimum fragment from Inv binding to integrin $\beta_1\alpha_5$) (Leong et al., 1995) and fibronectin. The binding of these proteins would compete for the I-Domain (or αA domain) binding pocket of the alpha subunit with the Cag apparatus proteins, possibly even with CagL, and inhibit the CagA translocation. The results summarized in Table 3-1 showed that there was no effect on the capacity of translocation of CagA under these conditions. At the same time, by testing CagA translocation in different cells lines, it was observed that this succeeded regardless of their cellular background (epithelia (AGS, GE11β), fibroblast (GD25β) or hematopoietic (HL-60, Jurkat). Different cellular backgrounds increased the chances that different sets of α subunits are expressed with the integrin β1 subunit. These data indicated that the interaction with the cells integrins could be limited to the β subunit.

Assuming that this interaction is between the Cag apparatus and the integrin β1 subunit extracellular part, Y2H assays were performed in order to identify the interacting partners of the apparatus. These assays identified the Cag components CagA (N-terminal), CagY (C-terminal) and CagI as possible interaction partners of the integrin β1 subunit. The lack of

interaction with CagL, suggested that CagL did not seem to be interacting with the integrin β1 extracellular domain. These results were expected, since the RGD binding partner is not mainly on the β subunit, but on the A domains of the α subunit of the integrin heterodimer. The Y2H interactions were successfully confirmed with pull down experiments using soluble integrin $\beta_1\alpha_5$ and integrin $\beta_1\alpha_1$, as well as with GST fusion proteins of CagA (full size), CagYc and CagI and their specific binding to cells containing integrin β1 (Figures 3-14, 3-16, 3-17C, 3-18B and 3-19B). After it was established in our lab that CagL does not play a role in the integrin β1-Cag apparatus interaction, the group of Steffen Backert (Kwok et al., 2007) published that CagL is essential for the functionality of the apparatus. They claimed that the necessity of CagL depends on its RGD motif, which allows an interaction with the integrin $\beta_1\alpha_5$ on AGS cells. These data contradict the experimental data presented here. In order to clarify this situation, P12 bacteria were generated in which *cagL* was deleted and mutants were complemented with wild type *cagL* (*cagL* RGD) and CagL forms lacking partial or the whole RGD domain (*cagL* RAD and *cagL*ΔRGD). As presented in figure 4-1, the RGD domain from CagL is neither necessary for CagA translocation nor for IL-8 induction. Since the results claiming the necessity of an RGD domain on CagL were not reproducible and the data obtained in the time of this thesis support that RGD does not play an essential role in CagA translocation, no further experiments followed in order to revalidate the published data.

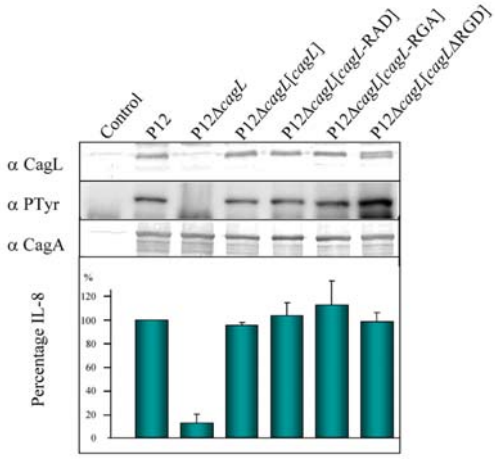


Figure 4-1 RGD domain from CagL is not essential for CagA translocation

Immunodetection of CagL, CagA and phosphorylated CagA after infection of AGS cells with P12 strains wild type and *cagL* strain mutants. These mutants were complemented with *cagL* containing the wild type RGD motif, substitution of the glycine (*cagL RAD)* and deletion of the RGD region (*cagL*Δ*RGD)*. IL-8 data from each of the infections. The experiments were done three times. The IL-8 is represented by the percentage in comparison to the wild type infection.

As said before, Cag apparatus proteins CagA, CagI and CagY are interacting directly with the integrin β1 subunit. Which of the integrin β1 domains are relevant for this interaction? Knowing the exact domain could give an idea of how the Cag apparatus exploits the binding to integrin β1 to translocate CagA into the cell.

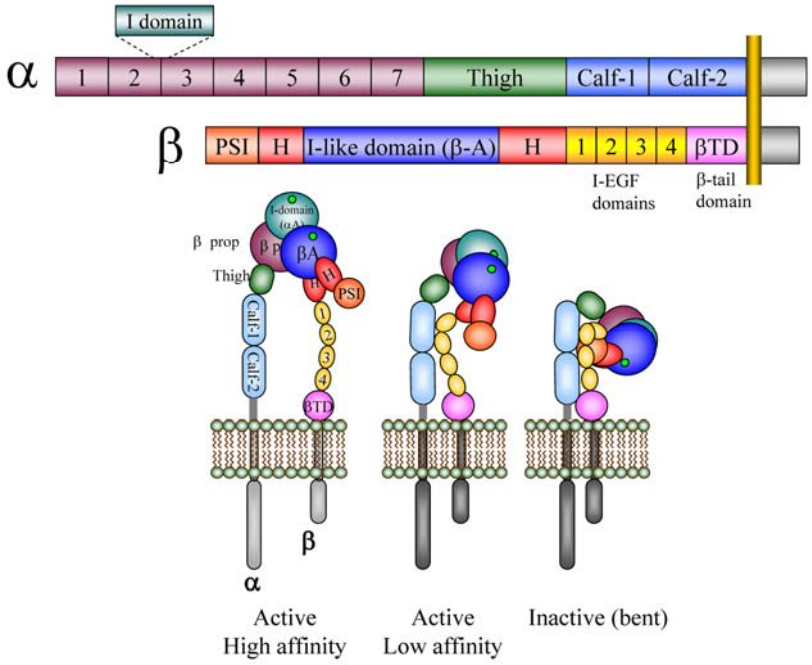


Figure 4-2 General domains of integrin and their activation status

Integrin alpha subunit contains a β propeller in which the I-domain (or αA domain) is found. They are followed by the Thigh domains and the Calf1 and Calf2 domains connected to the membrane through the transmembrane domain. The β subunit contains a PSI domain, followed by the I-like domain or the βA domains found between the hybrid domains. A highly cystein rich domain (EGF 1.4) attached to the hybrid domain connects the βTD to the rest of the extracellular part of the integrin β subunit. The three possible conformational states of the integrins, with different affinities and their structural movement (bottom).

Each of the domains in the integrin β subunit is important for the function of the integrin heterodimers. The function of the integrin β subunit is accomplished through specific interactions with the α subunit. The domains are represented in figure 4-2. In the structure of integrins, as mentioned before, both subunits, α and β, are transmembrane proteins. On the integrin β subunit at the N-terminal part the PSI (Plexin, Semaphorins and integrins) domain can be found. This domain is usually not accessible in inactivated integrins, only in activated forms (see figure 4-3). Activated integrins are able to bind to their ligands though the binding pocket formed by the I-domain of the α subunit supported by the I-like domain of the β subunit. The α subunit contains a bivalent-cation-binding domain at the N-terminal part, in the region denominated αA domain or I-Domain (Lu et al., 1998; Oxvig and Springer, 1998). In the I-like domain of the β subunit, Mg^{2+} and Ca^{2+} bind and activate/inactivate the integrins.

The binding of integrins to their ligands is always bivalent-cation dependent. This means that integrins are stimulated to bind to their ligands by Mg^{2+}, and are partially inhibited by Ca^{2+}, as shown for the first time for the heterodimer integrin $\beta_1\alpha_5$ (Gailit and Ruoslahti, 1988).

Since the Y2H assays showed to be a reliable way to explore integrin β1 with Cag apparatus interactions, a new Y2H assay was designed, in which the different domains of integrin β1 were scanned for interactions with the Cag proteins as mentioned before (Figure 3-21). The results of this assay, in conjunction with the fact that CagA translocation could be inhibited only by one antibody against integrin β1 (Figure 3-22), has led to the construction of the model presented in figure 4-4. In this model, the apparatus is able to bind to the activated form of integrin β1. This hypothesis is supported by the results showing higher CagA phosphorylation levels in conditions where integrin β1 is activated through exposure to $MnCl_2$, which is reported to act similar to Mg^{2+}, activating integrins by binding to the MIDAS (Metal Ion-Dependent Adhesion Site) pocket in the I-like domain of integrin β1 (Mould et al., 2002) (Figure 3-9B). The possibility that the Cag apparatus binds to an activated form of the integrin β1 is supported as well by the fact that CagYc, whose interacting domain partner seems to be the PSI domain, is able to bind with higher affinity to the integrin β1 expressing cells (Figure 3-23D) when Mn^{2+} is present. The presence of Mn^{2+} induces the extension of the integrins complex leaving the PSI domain of the β subunit accessible for the GST-CagYc to bind to the integrin β1.

The binding of CagA to the EGF-like domains (EGF (1-4)) and the β-tail domain (βTD) region as well as the binding of CagI to the βTD, are based on the Y2H assays results, but further experimental data needs to be done to confirm these observations. CagA was confirmed to be located on the surface of the apparatus, on the area around the tip, putting it on the right place for interaction with integrin β1, as it is shown in the EM immunogold pictures in figure 3-20.

In the model here, the necessity of a "bendable" form of the integrin β1 subunit for CagA translocation has been derived from the results obtained with the use of the anti-integrin antibody clone 9EG7.In this case the antibody is able to successfully block the CagA translocation in a binding dependent manner (Figure 3-22B & 3-22C), without inhibiting the binding of CagA, CagYc or CagI (Figure 3-23A). Although 9EG7 antibody recognizes an overlapping region of the integrin β1 that was identified via Y2H to be the possible binding domain of CagA N-terminal half to the integrin β1 (domains EGF (1-4) and part of the β-

TD), there was no competition between both of them. The fact that competition between the GST-fusion proteins and 9EG7 does not take place, indicates that the inhibition of CagA translocation by the 9EG7 antibody could be related to a conformational blockade of integrin β1 caused by the antibody. The 9EG7 antibody binds to human integrin β1 under activating conditions, which can be induced by either Mn^{2+} or Dithiothreitol (DTT) (Mould et al., 1995) (Ni et al., 1998) and is therefore associated to be a conformational epitope (Bazzoni et al., 1995). Several crystal structural studies, as well as EM negative staining studies, have revealed a bend/extended conformation change associated with the inactive/active form of the integrins (Arnaout et al., 2007). Most of these studies have been done on heterodimers containing integrin β3, but it has been accepted as a standard model for the integrin family members containing the same domain regions, as it is the case for integrin β1 heterodimers. In the "bend" or inactive form, the structure is completely bent on the "knee" region located on the β subunit between the EGF1 and EGF2 domains and stabilized in this bended form by interactions between the PSI-Hybrid-EGF and EGF (2-4)-βTD. Several contacts have been observed as well between the EGF (1-4) and βTD domains of the β subunit with Calf 1 and Calf 2 domains of the α subunits (Xiong et al., 2001), through the disulphide bridges between their cysteins (Kamata et al., 2004) (See Figure 4-2 for the bend and extended forms). In this latter region, the conformational epitope has been mapped and identified by 9EG7 antibody upon activation of the integrin β1. It is plausible that the binding of the antibody in these regions blocks the formation of the bridges and thus blocking the integrin in the extended form. This conclusion is supported by the PhD thesis of Michael Kristin while he was working with the biophysical properties of Focal Adhesions at the Georgia Institute of Technology (2006). If the binding of this antibody can cause a blockade of the bending of integrin β1, it would mean that this molecular movement is required by the Cag Type IV Secretion System, with the objective to get closer to the membrane.

From all other antibodies tested, none had an effect on CagA translocation, even though some like AIIB2, 12G10 and 8E3 are reported to affect the affinity of the integrin β1-containing heterodimers to their ligands (Takada and Puzon, 1993) (Mould et al., 1995), indicating that the I-domain, containing the ligand binding pocket, is not essential for CagA translocation. This data correlates with the lack of competition between the Cag apparatus and the I-Domain of the alpha subunit.

Experiments were performed as well with antibodies directed against the PSI domain, N29 and 8E3. Both epitopes recognized by them are found on the inside of the PSI domain, which

faces the α subunit (Mould et al., 2005). Knowing this, it is not surprising that these antibodies were not able to block the CagA translocation. Considering the model proposed (see in Figure 4-3) the antibodies could not compete with the CagYc of the apparatus because they would have a different binding region. At the same time, the study of the effects of these antibodies by Mould et al., indicates that their efficiency to stimulate activation is present only when the Calf-2 domain is absent. These results can be interpreted as a hierarchy of activation/inactivation motifs, in which the Calf-2 domain interaction with the EGF (2-4)-βTD has a higher control of the activation than the PSI domain. This correlates at the same time with our model based on the fact that only the 9EG7 antibody inhibits the CagA translocation.

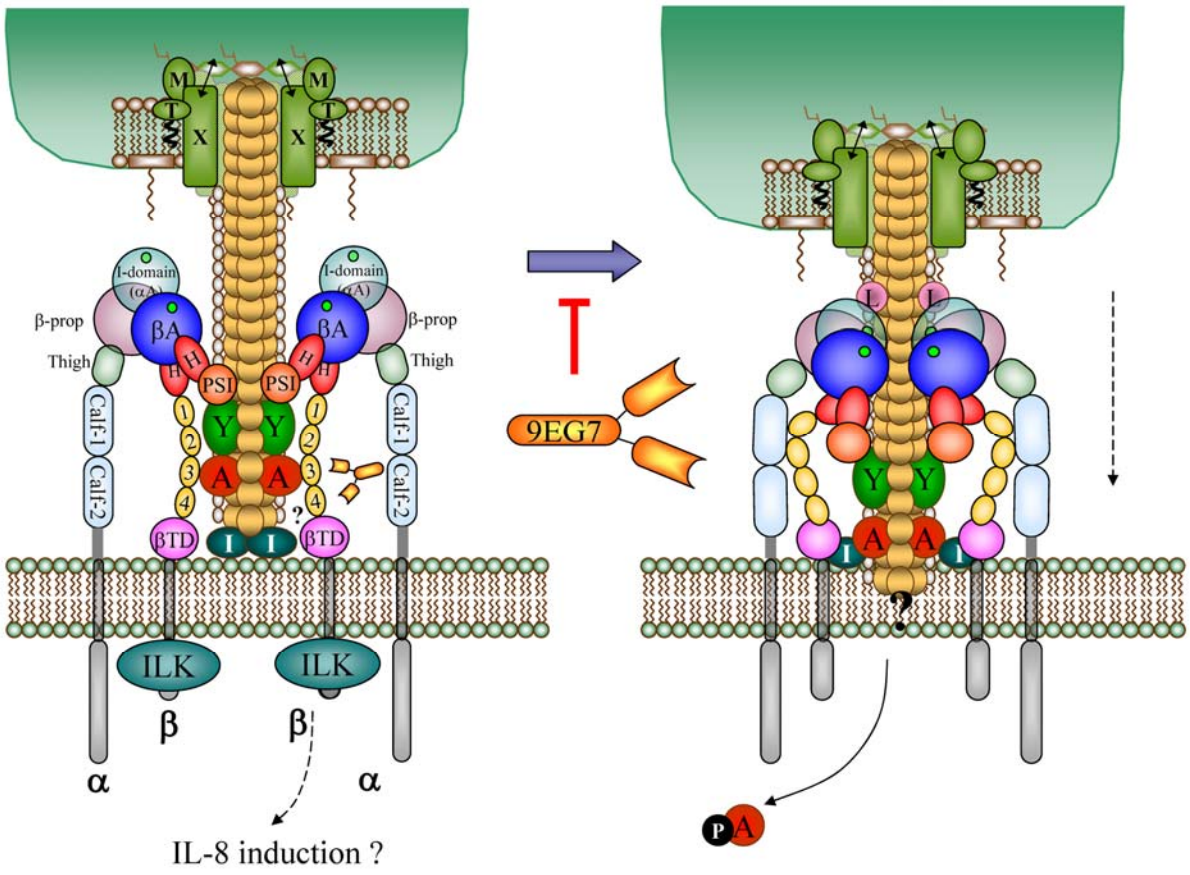


Figure 4-3 Model for Interaction of Cag apparatus with integrin β1.

Represented are the actual models of the Cag apparatus and the interactions of CagY, CagA and possibly CagI with the PSI, EGF and βTD domains of integrin β1 respectively. The binding of the apparatus or the apparatus itself, induces the bending necessary to get the pilus-like structure closer to the membrane. The blocking of this bending of integrin β1 could be the mechanism of action of the 9EG7 antibody.

4.3 Relevance of cellular processes for CagA translocation

Integrins function is dependent on a successful clustering (Vitte et al., 2004). Normally, clustering takes place in microdomains in the cell membrane named lipid rafts. Lipid rafts or microdomains have been defined as regions in the cell membrane rich in Sphingolipid:cholesterol composition, resistant to extraction in detergent conditions (1% Triton, 4°C) (Brown and London, 2000; Brown and Rose, 1992). The presence of cholesterol in these regions confers to the cell membranes special flexibility, that allows the curvature necessary for internalization events (Mukherjee and Maxfield, 2004). Even though transmembrane proteins are not usually associated with cholesterol, association of transmembrane proteins with lipid rafts have been found in cases where the proteins undergo an oligomerisation process (Cherukuri et al., 2001). In the case of integrin, binding events are not dependent on the cholesterol levels of the membrane, but cholesterol seems to be indispensable for signaling events related with the cytoskeleton as demonstrated for integrin LFA-1 ($\alpha_L\beta_2$) and $\beta_1\alpha_4$ (Leitinger and Hogg, 2002). Since the important role of integrin β1 in CagA translocation has been established, it is necessary to explore cellular processes related with the function of integrins. During determination of the integrin functions required for CagA translocation, the irrelevance of the cytoplasmic domain of the integrin β1 and the associated ILK pathway for CagA translocation has been recognized. However, because of their localization imbedded in the lipid bilayer, the transmembrane region does not only play a role in the anchoring of the integrins to the membrane. The conformational changes, associated with their inside-outside and outside-inside signaling, could have an effect on their association with the lipids in the membrane for transmitting signals to the cytoplasm. To date, more research has been done to discover this relation (Arnaout et al., 2007). A direct way to search for the necessity of the transmembrane domain and its effect on clustering for CagA translocation was the disruption of the lipid content in the membrane of the cells. The result of cholesterol depletion by a Methyl-β-Cyclodextrin (MBC) of AGS cells, conferred the cells with an apparent resistance to CagA translocation (Figure 3-30A). This resistance could be associated with the incapability to cluster the integrins by lipid raft association. However , these results could be an indirect consequence on the recruitment and not on the enzymatic activity, of Src kinases to the membrane. Src kinases are recruited to the membrane through their myristoylated and palmitoylated moiety that gives these kinases a higher preference to the ordered lipid domains in the membrane (Buss et al., 1986; Robbins et al., 1995). Since

CagA seems to have a membrane preference (Higashi et al., 2005), the phosphorylation would not take place in cholesterol-depleted cells if the Src recruitment were as well disturbed.

In order to confirm the relevance of clustering, calpeptin, a Calpain inhibitor, was tested (Figure 3-30C). Calpain is a cystein protease, and its activity has been related with the function of integrins on migration (Calle et al., 2006). The Calpain protein, a calcium dependent protease; plays an important role in the disassembly of focal contacts where integrins are linked to the cytoskeleton. Calpain can act by cutting the cytoplasmic tails of integrins, which is important for the formation of new small clusters, and create new focal adhesions (Huttenlocher, 2005) or by digestion of Talin, whose products had been shown to induce the clustering of β3 integrins (Ginsberg et al., 2001). By inhibiting the function of Calpain, clustering of integrins is reduced, or even blocked as demonstrated in motility effects on T-lymphocytes (Stewart et al., 1998). The fact that Calpain inhibition, through use of calpeptin, blocks CagA translocation could be associated with the necessity to cluster of integrin β1. The activity of Calpain is calcium dependent and it can explain the effect seen by the use of BAPTA, a membrane permeable calcium chelator. Pre-treatment of AGS cell with BAPTA can almost abolish the CagA phosphorylation (Figure 3-9). In an excellent study by Marlink et al., it was shown that one of the earliest events associated with a functional Cag apparatus was the release of intracellular calcium stocks from the Sarcoplasmic/Endoplasmic Reticulum, with the help of the Sarcoplasmic/endoplasmic reticulum Calcium ATPases (SERCA) (Marlink et al., 2003). It is possible that the first effect of Cag apparatus components, triggered by binding to the integrin β1, induces an elevation of calcium concentration in the cytoplasm to induce the activity of calpains and therefore allowing the clustering of the integrins.

If clustering of integrin β1 is necessary for CagA translocation, the possible following effect caused by the Cag apparatus could be the initiation of internalization by endocytic processes. In general, cells are able to take up molecules from the environment through different endocytic processes. These processes include the involvement of proteins like caveolin, clathrin, flotillin or actin (Miaczynska and Stenmark, 2008). Their function is to support and allow the deformation of the membrane in order to engulf particles and take them inside the cells. Since all of them have been associated with a functional actin polymerization, its necessity for CagA translocation was evaluated. In the case of *H. pylori* CagA translocation, the exposure of cells to cytoskeleton disruptors, such as Cytochalasin D or Jaxplakinoline, showed no defect on the CagA translocation and phosphorylation (Figure 3-30A and Table-

1). Since microtubules are involved in the transport of vesicles from the membrane towards the inside of the cells, microtubule disruptor Nocodazole was tested as well, giving the same results (Table 3-1). Based on these results, it was assumed that endocytic processes do not take part in the CagA translocation. However, reports claiming that the cytoskeleton is not essential for a successful clathrin dependent endocytic process (Kirchhausen, 1999; Mousavi et al., 2004; Rappoport et al., 2004) reopened the question of endocytosis and CagA translocation. To be able to rule out completely the possibility that endocytic processes are related with the CagA translocation, two inhibitors of clathrin were used: Mono-dansylcadaverine (MDC) and Dynasore. Each inhibitor disturbs a different part of the clathrin dependent endocytosis. MDC is claimed to inhibit the recycling of clathrin for the formation of new Clathrin Coated Pits (CCP) and therefore, Clathrin Coated Vesicles (CCV) are not formed (Phonphok and Rosenthal, 1991). Dynasore, on the other hand, is a specific inhibitor of the ATPase activity of Dynamin (Macia et al., 2006). Dynamin is a protein associated with the recruitment of clathrin molecules to membranes as well as with the final excision of CCP from the membranes to form CCVs (Rappoport et al., 2003) (Ehrlich et al., 2004) (Roux et al., 2006). As presented in figure 3-33A and 3-33B, the use of these two inhibitors shows that CagA translocation requires an intact clathrin pathway, since no CagA phosphorylation is detected in their presence. Any possible inhibitory effect on the bacteria could be ruled out in time experiments or by direct exposure of bacteria to the inhibitor (Figures 3-33C and 3-33C). These results implied that the CagA phosphorylation requires clathrin or clathrin components for CagA to be translocated into the cytosol. If a successful uptake via CCV is necessary for CagA translocation, the question arises, what mechanisms are necessary for the CagA transport from the lumen of the clathrin formed vesicle into the cytoplasm in order for CagA to be phosphorylated by Src kinases?.

Fibroblast Growth Factor 1 (FGF-1) is able to escape the lumen of endocytic vesicles and to enter the cytoplasm. In the studies by Malecki et al. (Malecki et al., 2002) several methods are described to examine if extracellular added FGF-1 is translocated into the cytoplasm after endocytosis through events linked to acidification of the endosome or to changes of vesicle membrane potentials. They used ammonium chloride (NH_4Cl) and Monensin A (Na^+/H^+ antiporter, inhibits acidification of endosomal lumen), as well as Bafilomycin A and Concanamycin A, both vacuolar proton pump inhibitors. In their study, they found that FGF-1 was blocked by treatments that altered the membrane potentials. In a following paper, they discovered that the translocation effect was supported by Hsp90 (Wesche et al., 2006), a chaperone protein that has been associated as well with the IL-8 induction by *H. pylori* Cag

apparatus (Yeo et al., 2004). To evaluate if CagA translocation into the cytoplasm uses a similar mechanism as the FGF-1, infections were done in presence of Monensin A, Concanamycin A (inhibitor of vacuolar ATPases), Bafilomycin A (inhibitor of Vacuolar Type H ATPases), Ammonium Chloride (inhibits acidification of endosomal lumen) and Geldanamycin (Hsp90 specific inhibitor). Neither of the disrupted processes (the acidification of the lumen of endosomes, disruption of changes on membrane potentials and the inhibition of Hsp90) were able to inhibit the CagA phosphorylation (Figure 3-34 and Table 3-1). The question which mechanism "triggers" the translocation of CagA from the lumen of endosomes to the cytoplasm could not be answered. It is possible that the apparatus uses an unknown mechanism to transport CagA from the lumen of endosomes to the cytoplasm, or another role of the clathrin pathway has to be considered. Further information and research on toxins and viruses could give an idea which other processes are required for the escape of proteins from the endocytic lumen into the cytoplasm, in order to elucidate the mechanism of CagA into the cytoplasm.

The fact that clathrin or some of the components associated with its pathway are needed for the translocation of CagA by the Cag apparatus had to be confirmed visually through immunofluorescence detection of clathrin and Cag apparatus components. After infection with GFP-expressing P12 wild type strain and the *cagPAI* mutant P12 Δ*cagPAI*, detection of clathrin confirmed a co-localization event of clathrin molecules with bacteria in a Cag-dependent manner (Figure 3-36A). Furthermore, double immunostaining against clathrin and CagY showed some co-localization events between these two proteins, relating the presence of a Cag apparatus with clathrin-rich areas. These results could add *H. pylori* to the new list of bacteria using the clathrin pathway, as recently discovered for *Listeria monocytogenes*, which uses clathrin dependent endocytosis to enter the cells (Bonazzi and Cossart, 2006). Many other pathogens use clathrin to gain access into the cells, as it is the case for some viruses like Hepatitis C (Helle and Dubuisson, 2008) or Adenoviruses (Meier and Greber, 2004). Because of this common "necessity" of getting into the cell, it makes the use of this pathway a plausible option for viruses, toxins or facultative intracellular bacteria. In the case of *H. pylori*, bacteria do not seem to be keen in entering the cells. Could it be that they use clathrin only to get CagA inside the cell? And if it is so, how? Is there a protein from the Cag apparatus that functions as "translocating" protein once they are found in the endosomal compartment? What could be the possible trigger for this event to occur? With the results presented here, it can be said that the acidification and/or changes of the transmembrane potential in the endosomes are not required. Further studies are necessary to elucidate the

mechanisms involving the necessity of clathrin endocytosis and its components for the CagA translocation.

4.4 IL-8 induction and CagA translocation are two different events

A functional Cag apparatus is not only able to translocate CagA into the eukaryotic cell. Its functionality has been linked to the capacity of inducing the production of IL-8 in human cells. During the experiments performed trying to explain the interaction between the Cag apparatus and integrin β1, the capacity of IL-8 induction in different steps of this interface was evaluated. Figure 4-4 presents a proposed model of *H. pylori* interaction with integrin β1 and the effects associated with it: CagA translocation and IL-8 induction.

4.4.1 IL-8 and integrin β1

4.4.1.1 Role of ILK pathway

By evaluating the necessity of a signal transduction process by the integrin β1 to ILK, in which no effect was seen on CagA translocation, it was surprising to find out that the capacity of IL-8 induction by the Cag apparatus was abolished, leaving only small induction levels. This small induction can be associated to the effective CagA translocation and phosphorylation, since it has been shown that CagA in the cell is responsible for some levels of IL-8 induction (Brandt et al., 2005) (Chang et al., 2006). Previously IL-8 induction via the Cag apparatus had been associated to the activation of NOD1 by the peptidoglycan from *H. pylori*. It was assumed that peptidoglycan was able to enter into the cytoplasm of cells when it "crossed" the cellular membrane for the injection of the CagA protein. Once in the cytoplasm, protein NOD1 signaling was responsible for IL-8 induction associated with the Cag apparatus (Viala et al., 2004). With the results obtained here, the relevance of the NOD1 signaling, as only signal pathway to be responsible for IL-8 induction, should be reconsidered. Even though CagA has been successfully translocated in ILK deficient AGS cells, implying peptidoglycan translocation into the cytoplasm of the cells, no IL-8 was induced at the levels usually seen for intact systems in ILK deficient cells.

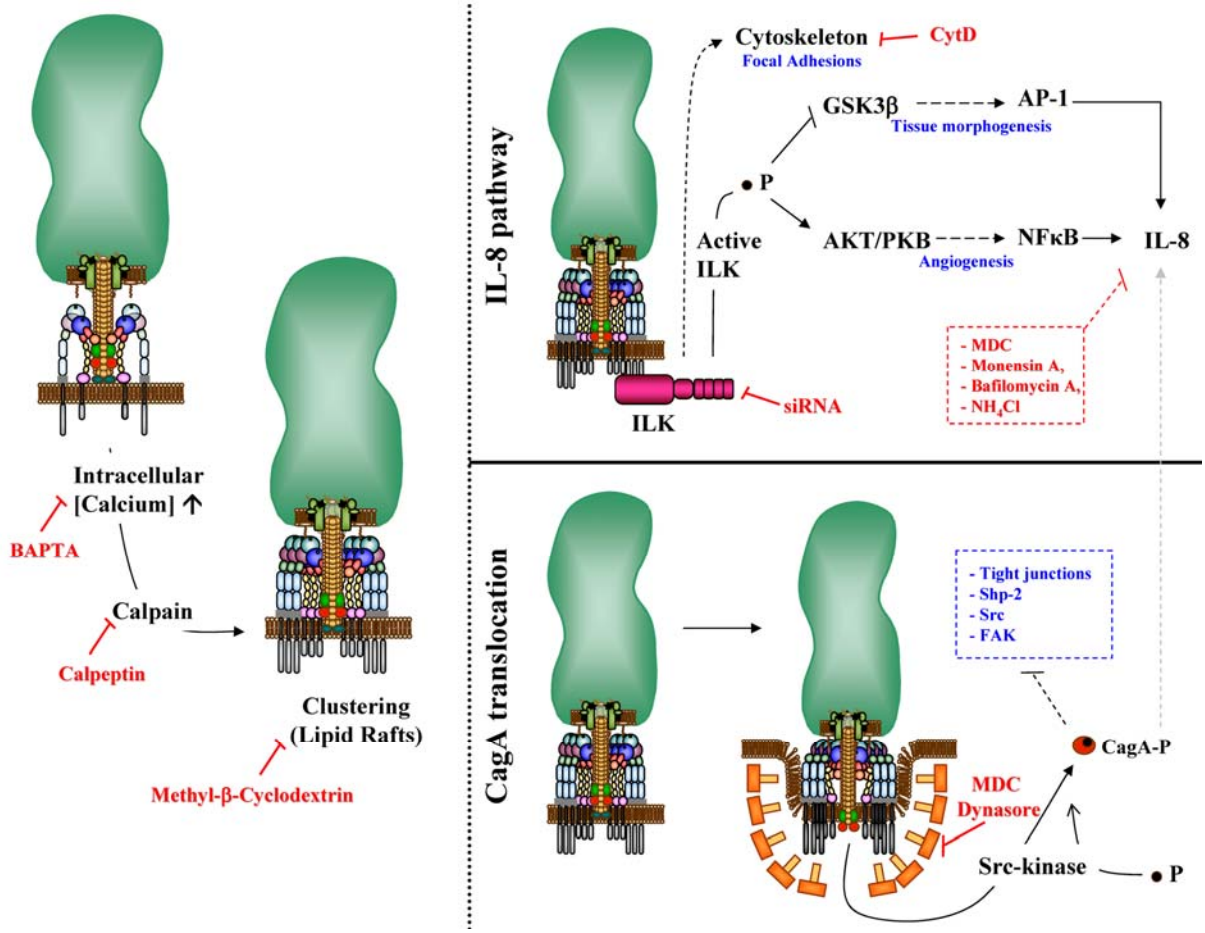


Figure 4-4 Final model for Cag apparatus interaction with integrin β1.

Upon Cag apparatus contact with the integrin β1, an elevation of intracellular calcium takes place, activating the calpains and inducing integrin aggregation. Both effects related to Cag apparatus functionality are independent from each other. IL-8 induction might be induced upon activation of the integrin linked kinase (ILK), which can phosphorylate the AKT/PKB pathway and indirectly the NFkB translocation into the nucleus (Shown for Angiogenesis). ILK can as well inactivate the GSK3β by phosphorylation leading indirectly to the translocation of AP-1 to the nucleus. Both components AP-1 and NFkB are necessary for IL-8 induction. CagA translocation might require the clathrin or clathrin components at the point of contact to stabilize the "pore", or to induce endocytosis of CagA for later translocation. The inhibitors and their targets are shown in red. The associated processes are shown in blue.

4.4.1.2 Effect of integrin β1 activation

CagA translocation and IL-8 induction are independent events. This is supported by the fact that IL-8 induction is severely reduced in conditions were Mn^{2+} is present (Figure 3-27), which is capable to constitutively activate integrin β1. These data are similar to the data obtained with ILK deficient cells. When cells were exposed to $MnCl_2$, CagA translocation was more efficient compared to CagA translocation in its absence. However, the IL-8 induction was inversely proportional to the CagA phosphorylation increment.

Both experiments support as well the possibility that the IL-8 induction is directly connected with the capacity of the Cag apparatus to bind and modify the structure of integrin β1. IL-8 induction via integrin β1 is commonly known. It has been proven that binding of invasin to cells induces the production of IL-8, and that this induction is independent of the internalization effect triggered by its interaction with integrin $\beta_1\alpha_5$ (Schulte et al., 1998). In their experiments, the phosphatydylinositol-3 kinase (PI3-K) was blocked using Wortmannin, a fungal product that specifically inhibits PI3-Ks. The same inhibitor was tested for IL-8 induction via the Cag apparatus (Figure 3-35) obtaining similar results. However, the data showing a defective IL-8 induction when ILK-levels are reduced would support a possible pathway between integrin and IL-8 induction. As explained by Legate KR et al. (Legate et al., 2006), the integrin β1 - ILK pathway activates the Akt/PBK pathway that induces the translocation of NFkB into the nucleus. Since IL-8 induction requires NFkB, both pathways are similar and not excluding each other (Figure 4-4).

4.4.1.3 Role of integrin extracellular domains

If integrin β1 is directly related with IL-8 induction via Cag apparatus, the effect should start at the moment there is contact between Cag T4SS and the extracellular part of integrin β1. For that, antibodies against the different domains of integrin β1 were used to evaluate their effect on IL-8 induction. As summarized in figure 3-29, AIIB2, which inactivates the capacity of integrin β1 to bind to their substrate, worked as agonist of IL-8 induction in infections of cells exposed to the antibody. The levels of IL-8 induction are almost doubled compared to normal conditions. This effect could be associated with the increment of available integrins for the apparatus to "dock on" as they lost attachment. Since the binding of this antibody does not have any effect on CagA translocation, the independence of IL-8 and CagA phosphorylation events is demonstrated. This independence can be recognized better by examination of the effects of IL-8 in presence of antibody clone 9EG7. This antibody is the only one able to inhibit CagA translocation. However, it has only a very small effect on IL-8 induction, not proportional with the CagA phosphorylation effect seen earlier. This IL-8 reduction could be associated with the lack of CagA in the cytoplasm of the cells, as explained for ILK siRNA effects on IL-8 before. The data collected with the other antibodies, show a relation of antibody epitope localization on integrin β1 and IL-8 induction. The closer the antibodies' epitopes got to the transmembrane region, the higher the effect on IL-8 reduction. At the same time, the antibody clone LM534, defined as a neutral antibody, was able to induce IL-8

without the presence of the bacteria. The induction by the Cag apparatus in presence of this antibody was dramatically reduced, as the apparatus and the antibody could use the same mechanism. In previous experiments not shown here, IL-8 induction by *H. pylori* was analyzed in presence of PMA. In this case, even though both, bacteria and PMA were able to induce IL-8 production, PMA did not reduce the IL-8 induction. It actually appeared as PMA and *H. pylori* effects on IL-8 were additive, what would indicate two different mechanisms. Further experiments using only the F(ab') parts of the LM534 can elucidate if a crosslinking effect of the βTD of integrin β1 could be the cause of this induction and therefore also the mechanism used by *H. pylori* to induce IL-8.

4.4.2 Involvement of cellular processes in IL-8 induction

Together, these results indicate that CagA translocation and IL-8 induction are two independent events on the cell, even though both are related with Cag apparatus functionality. At the same time, this data opens the possibility that the interaction with the integrin is the possible trigger for IL-8 induction. Keeping this possibility open, IL-8 induction via Cag apparatus was evaluated under depletion of cholesterol from the membrane. The disruption of lipid rafts had a surprising effect on IL-8 induction. In this case, the effect seems to be strain specific, as for P12, there was an agonist effect, while strain 26695 showed an inhibitory effect (Figure 3-31). The difference cannot be explained, since both strains presented a reduction in CagA translocation when cells were treated with MBC. These data confirm that IL-8 induction and CagA translocation are different events in the process of CagA translocation and Cag interaction with the cell. However, it does not explain the role of integrin β1 clustering in IL-8 induction.

Changes on the topology of the membrane can be achieved by the use of proteases like thrombin and trypsin. These two proteases are able to digest several extracellular epitopes of transmembrane proteins. On the other hand, integrins and other growth factor receptors are resistant to digestion by these proteases. To evaluate the possibility that a second receptor associated with integrins is responsible for the functionality of the Cag apparatus, pre-digestion of extracellular proteins was followed by infection with different *H. pylori* wild strains containing a functional Cag apparatus. The treatment did not have any visible effect on CagA phosphorylation after a 4 hours infection (Table 3-1). In IL-8 induction, however, alone the presence of trypsin increased 10-fold the amount of IL-8 in comparison with untreated cells. Since trypsin induces detachment of cells from the surface, the effect of detachment was

analyzed. This showed a significant difference in comparison with only the presence of trypsin. The effect of detachment can be explained with the fact that the amount of integrins available to interact with the Cag apparatus is higher than that on the attached cells. The effect of trypsin digestion increasing the IL-8 induction was, on the other hand, unexpected. Considering that integrin β1 is resistant to trypsin digestion (Sczekan and Juliano, 1990), one way that cells could be stimulated by the use of a protease would be through activation of PARs, Protease Activated Receptors. PARs are a family of seven transmembrane spanning proteins that are activated by cleavage of their amino-terminal exodomain by proteases like trypsin (PAR4) and thrombin (PAR1). As with other transmembrane domains, their activation induces the phosphorylation of their carboxyl-terminal YSIL domain and induces the recruitment of clathrin Adaptor Proteins (AP-2) to be endocytosed (Trejo, 2003). It will not be surprising that their activation would involve integrin β1 and in this way influence the IL-8 response by the cell to the Cag apparatus.

The effects on IL-8 observed as the endocytic processes were disrupted cannot be considered sufficiently reliable to determine a relationship between Cag apparatus and the cell. The secretion of IL-8 into the supernatant can be inhibited unspecific during exposure of the cells to these inhibitors.

The role of Cag apparatus in IL-8 translocation had been examined here. It can be concluded that the events “CagA translocation/phosphorylation” and “IL-8 induction” associated with a functional Cag T4SS are two independent events. CagA translocation can take place, without inducing IL-8 in the levels expected for a functional apparatus, and IL-8 can be induced without CagA translocation as well.

4.5 The composition of the Pilus-like structure of the Cag apparatus

During live imaging recording of infection events, a specific and conserved behavior of bacteria was observed. Bacteria strains containing flagella (P12, P145 and P217) showed a preference for cells that had lost adhesion. As the trypsin experiments caused the loss of adhesion of the cells, it was observed that this bacterial tropism was not present anymore. Their preference was limited to those cells showing a signal of apoptosis, such as “blebbing” of their membrane or increment of auto-fluorescence observed after excitation with a 488 nm wavelength light source. This behavior, additionally to the fact that CagA translocation and

the related event "Hummingbird phenotype" increased in conditions where cells were stressed, indicated that a stress related unknown molecule could be activating and/or stimulating the Cag apparatus. Taking advantage of this knowledge, and with the necessity to increase the amount of bacteria expressing the already pilus-like structures to be associated with the Cag apparatus (Rohde et al., 2003) for Cryo-EM studies, AGS cells were exposed to a PBS solution without bivalent cations. This procedure stresses the cells and makes themsecrete a possible substance able to induce the Cag apparatus formation. It was discovered that after the exposure of bacteria to this exudate, the amount of apparati increased about 20% - 25% compared to those only in the PBS solution.

The pilus-like structures formed during the treatment could be confirmed to consist of two different structures: A sheathed form and a naked one. It looks like the sheathed form is composed of naked form (inside) covered by a molecule that has not been identified yet. However, it could be shown that the naked form contains CagC protein, making this pilus-like structure part of the *H. pylori* T4SS. The naked form seems to be formed by oligomers assembled to construct a tubular structure with 2 - 3 nm of diameter in the inside and 20-25 nm of diameter on the outside. The sheath covering the naked core form, who's molecular composition has not been identified, does not show any repetitive structure.

For *A. tumefaciens*, the VirB system has been observed with a pilus-like structure with 10 nm diameter. Its formation is promoted by the VirB1 and on its surface is found the VirB5 (Aly and Baron, 2007; Zupan et al., 2007). However, in the EM images only one structure having as major component the VirB2 protein was observed. Khaled et al. were able to detect VirB2 by immuno-EM only on detached T-pili, but they were not bound to the bacteria. In our case, the sheathed form, containing the core CagC structure could be observed getting out of the bacteria (Figure 3-38B). One of the genes found in the *H. pylori cagPAI* might encode a coating molecule to form the sheath. In ongoing research, this sheath will be identified and a better picture of the components of the Cag apparatus pilus-like structure will be elucidated.

5 Conclusions

Helicobacter pylori, as many other bacterial pathogens, contains within its virulence arsenal the Cag Type IV Secretion System. Epidemiological studies have revealed a correlation of the severity of gastric *H. pylori*-associated pathologies with the presence of CagA and VacA, but not with the presence of a functional Cag apparatus. The effect of CagA on the host cells has been related with its translocation into the cell by the T4SS. During the evaluation of possible effects of CagA in the motility of neutrophils-like cells, it was discovered that not only the presence of CagA was disturbing the motility towards a gradient, but also the possibility was opened that this effect seen is independent of the successful translocation and phosphorylation of CagA. If that would be the case, a new immunomodulating activity of CagA could be added to the many functions associated to this multifacetted toxin. It could explain why only CagA presence, and not a functional secretion system, is associated with disease.

While establishing the effect of the Cag T4SS on motility, the essentiality of integrin β1 for the translocation and phosphorylation of the CagA by the Cag apparatus was discovered. In previous studies, performed by the team of Prof. Dr. Rainer Haas, the theory of the necessity of a receptor on the cell membrane interacting with the T4SS for translocation of CagA was formulated. During this doctoral study, it has been determined that integrin β1 is the receptor for the Cag Type IV Secretion System and the signaling related to the integrin β1 is responsible for the IL-8 induction. In the case of CagA translocation, only the extracellular and transmembrane domains play an important role, what gives the integrin β1 the function of a "docking" molecule. It seems that the capability of integrin β1 to change conformations from extend to bend has made it a perfect "docking" molecule for the apparatus. Even though, neither the intracellular domains nor the ILK-related pathways of integrin β1 are relevant for CagA translocation, functions related with clustering seem to be relevant for the CagA translocation. Both functions, the capacity of bending and forming clusters, could play an independent but important role in the CagA translocation. While the binding to an activated form of the integrin β1 increases the efficiency of CagA translocation, as seen in the presence of $MnCl_2$, clustering of integrins could stabilize the binding of the apparatus to the cells. At the same time, the capacity of "bending" of the extracellular domain of integrin β1 could reduce the distance between the tip of the pilus-like structure of the apparatus and the cell membrane and facilitate the translocation of CagA. How this translocation occurs, is one of the many questions that have to be answered in future research.

Another discovery made was the involvement of clathrin dependent endocytosis in CagA phosphorylation. The participation of clathrin or clathrin associated proteins open the possibility of an endocytic process necessary for translocation. However, the failure to inhibit CagA translocation by disrupting endocytic transport by microtubules or actin, as well as endosome maturation related processes, as acidification and changes in membrane potential, makes it difficult to accept an uptake of Cag components necessary for CagA translocation. Another possibility to explain the involvement of clathin or clathrin associated proteinscould be the necessity of these proteins for the stabilization of the pore created by the Cag apparatus in order to get CagA inside the cell. Both theories should be considered in following studies relating the Cag apparatus interaction with the eukaryotic cell.

Knowing a little bit more about the behavior of *H. pylori* on the first minutes of infection and under different conditions, a way to stimulate the formation of a pilus-like structure without the presence of eukaryotic cells was found. The presence of such structures has been identified in past research to be related with the Cag apparatus. Knowing a way to induce their formation can help to produce enough material for Cryo-EM studies realized in conjunction with Dr. Valério Mathias at the Max-Planck Institute in Martinsried. These studies will not only determine the structure of the Cag apparatus and the components seen, they will also help to determine which proteins from the Cag apparatus are associated with its formation. Equally interesting will be to discover the molecule or molecules produced by the AGS cells that can induce the formation of the structure associated with the Cag T4SS.

It is only to hope, that the efforts done here to learn more about the interaction of *H. pylori*'s Cag T4SS will allow in the future a better understanding of the biomechanics by which pathogens, especially *Helicobacter pylori*, are able to deliver substrates into the cells. By understanding the minimal requirements for their success, the mechanism could be copied and used for targeted delivery of compounds to cancer cells, or even parasites hidden in the depths of an organism.

6 Acknowledgements

First, I would like to thank my husband. If it were not for your desire to be called “Herr Doktor”, I would have given up everything over a year ago. Honestly, without your patience and support I could not have made it. Without you, it would not have been possible to find a balance between you and work.

Second, I thank Rainer. It was a pleasure to have taken the road to develop into a scientist with your guidance. It was nice to have somebody that could dream with me about new crazy theories with the same passion. However, I will not forget the short vacations.

Wolfgang, you were not there for me only to help me filling in the “gaps” in my education and my selective memory about chemistry. You kept my scientific feet down in the moments of high dreams as speculations… thanks

To Tina, I am happy to have shared the first years in the lab with you. It was nice to find a good friend hidden behind an excellent scientist. I did miss you and your help in the last two years of my thesis work.

Xaver, I think it will be weird if I start to be nice with you… but I think it has to be in this moment. After all, it will be printed forever. Let it be a one-time occasion to thank you for your patience and for being an excellent student… sorry, friend…

Friederike and Evelyn, my transition from an american life to a german one would not have been possible without your friendship. I enjoyed all breakfast discussions and learned so much about Germany as you have learned from America and Colombia. Thank you for your patience and for your understanding in my pickiness about the cell culture.

Walther, my dear far-distance counselor. Thank you for teaching and encouraging me to be a scientist.

Isabelle, my dear desk-neighbor. I would have liked to have you for a longer time… our times of distress became only mine after you left.

Iris and Stephanie, it was nice to get new ears for my crazy theories. I thank you for your patience and for the opportunity that you gave me to be myself.

Claudia, all your new white hairs are my fault, but you have been a great support in this last 8 months of this work. Thank you for been honest with me, and REALLY read my thesis.

7 References

Ahmadian,M.R., Wittinghofer,A., and Schmidt,G. (2002). The actin filament architecture: tightly regulated by the cells, manipulated by pathogens. International Titisee Conference on the actin cytoskeleton: from signalling to bacterial pathogenesis. EMBO Rep. *3*, 214-218.

Aktories,K. and Barbieri,J.T. (2005). Bacterial cytotoxins: targeting eukaryotic switches. Nat. Rev. Microbiol. *3*, 397-410.

Al-Ghoul,L., Wessler,S., Hundertmark,T., Kruger,S., Fischer,W., Wunder,C., Haas,R., Roessner,A., and Naumann,M. (2004). Analysis of the type IV secretion system-dependent cell motility of Helicobacter pylori-infected epithelial cells. Biochem. Biophys. Res. Commun. *322*, 860-866.

Algood,H.M. and Cover,T.L. (2006). Helicobacter pylori persistence: an overview of interactions between H. pylori and host immune defenses. Clin. Microbiol Rev. *19*, 597-613.

Allen,A. and Garner,A. (1980). Mucus and bicarbonate secretion in the stomach and their possible role in mucosal protection. Gut. *21*, 249-262.

Alm,R.A., Bina,J., Andrews,B.M., Doig,P., Hancock,R.E., and Trust,T.J. (2000). Comparative genomics of Helicobacter pylori: analysis of the outer membrane protein families. Infect. Immun. *68*, 4155-4168.

Alm,R.A. and Trust,T.J. (1999). Analysis of the genetic diversity of Helicobacter pylori: the tale of two genomes. J Mol. Med. *77*, 834-846.

Aly,K.A. and Baron,C. (2007). The VirB5 protein localizes to the T-pilus tips in Agrobacterium tumefaciens. Microbiology. *153*, 3766-3775.

Arnaout,M.A., Goodman,S.L., and Xiong,J.P. (2007). Structure and mechanics of integrin-based cell adhesion. Curr. Opin. Cell Biol. *19*, 495-507.

Arnaout,M.A., Mahalingam,B., and Xiong,J.P. (2005). Integrin structure, allostery, and bidirectional signaling. Annu. Rev. Cell Dev. Biol. *21:381-410.*, 381-410.

Aspholm,M., Olfat,F.O., Norden,J., Sonden,B., Lundberg,C., Sjostrom,R., Altraja,S., Odenbreit,S., Haas,R., Wadstrom,T., Engstrand,L., Semino-Mora,C., Liu,H., Dubois,A., Teneberg,S., Arnqvist,A., and Boren,T. (2006). SabA is the H. pylori hemagglutinin and is polymorphic in binding to sialylated glycans. PLoS. Pathog. *2*, e110.

Aspholm-Hurtig,M., Dailide,G., Lahmann,M., Kalia,A., Ilver,D., Roche,N., Vikstrom,S., Sjostrom,R., Linden,S., Backstrom,A., Lundberg,C., Arnqvist,A., Mahdavi,J., Nilsson,U.J., Velapatino,B., Gilman,R.H., Gerhard,M., Alarcon,T., Lopez-Brea,M., Nakazawa,T., Fox,J.G., Correa,P., Dominguez-Bello,M.G., Perez-Perez,G.I., Blaser,M.J., Normark,S., Carlstedt,I., Oscarson,S., Teneberg,S., Berg,D.E., and Boren,T. (2004). Functional adaptation of BabA, the H. pylori ABO blood group antigen binding adhesin. Science *305*, 519-522.

Bagnoli,F., Buti,L., Tompkins,L., Covacci,A., and Amieva,M.R. (2005). Helicobacter pylori CagA induces a transition from polarized to invasive phenotypes in MDCK cells. Proc. Natl. Acad. Sci. U. S. A *102*, 16339-16344.

Bandyopadhyay,P., Liu,S., Gabbai,C.B., Venitelli,Z., and Steinman,H.M. (2007). Environmental mimics and the Lvh type IVA secretion system contribute to virulence-related phenotypes of Legionella pneumophila. Infect. Immun. *75*, 723-735.

Bazzoni,G., Shih,D.T., Buck,C.A., and Hemler,M.E. (1995). Monoclonal antibody 9EG7 defines a novel beta 1 integrin epitope induced by soluble ligand and manganese, but inhibited by calcium. J. Biol. Chem. *270*, 25570-25577.

Blaser,M.J. (1993). Helicobacter pylori: microbiology of a 'slow' bacterial infection. Trends Microbiol. *1*, 255-260.

Blaser,M.J. (1997). Not all Helicobacter pylori strains are created equal: should all be eliminated? Lancet. *349*, 1020-1022.

Boelens,M.C., van den,B.A., Vogelzang,I., Wesseling,J., Postma,D.S., Timens,W., and Groen,H.J. (2007). Differential expression and distribution of epithelial adhesion molecules in non-small cell lung cancer and normal bronchus. J Clin. Pathol. *60*, 608-614.

Bonazzi,M. and Cossart,P. (2006). Bacterial entry into cells: a role for the endocytic machinery. FEBS Lett. *580*, 2962-2967.

Brakebusch,C., Hirsch,E., Potocnik,A., and Fassler,R. (1997). Genetic analysis of beta1 integrin function: confirmed, new and revised roles for a crucial family of cell adhesion molecules. J Cell Sci. *110*, 2895-2904.

Brandt,S., Kwok,T., Hartig,R., Konig,W., and Backert,S. (2005). NF-kappaB activation and potentiation of proinflammatory responses by the Helicobacter pylori CagA protein. Proc. Natl. Acad. Sci. U. S. A *102*, 9300-9305.

Broussard,J.A., Webb,D.J., and Kaverina,I. (2008). Asymmetric focal adhesion disassembly in motile cells. Curr. Opin. Cell Biol. *20*, 85-90.

Brown,D.A. and London,E. (2000). Structure and function of sphingolipid- and cholesterol-rich membrane rafts. J Biol. Chem. *275*, 17221-17224.

Brown,D.A. and Rose,J.K. (1992). Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell *68*, 533-544.

Buss,J.E., Kamps,M.P., Gould,K., and Sefton,B.M. (1986). The absence of myristic acid decreases membrane binding of p60src but does not affect tyrosine protein kinase activity. J Virol *58*, 468-474.

Calderwood,D.A. (2004). Integrin activation. J Cell Sci. *117*, 657-666.

Calle,Y., Carragher,N.O., Thrasher,A.J., and Jones,G.E. (2006). Inhibition of calpain stabilises podosomes and impairs dendritic cell motility. J Cell Sci. *119*, 2375-2385.

Cascales,E. and Christie,P.J. (2003). The versatile bacterial type IV secretion systems. Nat. Rev. Microbiol. *1*, 137-149.

Cave,D.R. (2001). Chronic gastritis and Helicobacter pylori. Semin. Gastrointest. Dis. *12*, 196-202.

Chang,Y.J., Wu,M.S., Lin,J.T., Pestell,R.G., Blaser,M.J., and Chen,C.C. (2006). Mechanisms for Helicobacter pylori CagA-induced cyclin D1 expression that affect cell cycle. Cell Microbiol *8*, 1740-1752.

Cheng,Y., Macera,C.A., Davis,D.R., and Blair,S.N. (2000). Does physical activity reduce the risk of developing peptic ulcers? Br. J Sports Med. *34*, 116-121.

Cherukuri,A., Dykstra,M., and Pierce,S.K. (2001). Floating the raft hypothesis: lipid rafts play a role in immune cell activation. Immunity. *14*, 657-660.

Collins,S.J., Ruscetti,F.W., Gallagher,R.E., and Gallo,R.C. (1979). Normal functional characteristics of cultured human promyelocytic leukemia cells (HL-60) after induction of differentiation by dimethylsulfoxide. J. Exp. Med. *149*, 969-974.

Croft,D.N. (1967). Gastritis. Br. Med J. *4*, 164-166.

Cue,D., Southern,S.O., Southern,P.J., Prabhakar,J., Lorelli,W., Smallheer,J.M., Mousa,S.A., and Cleary,P.P. (2000). A nonpeptide integrin antagonist can inhibit epithelial cell ingestion of Streptococcus pyogenes by blocking formation of integrin alpha 5beta 1-fibronectin-M1 protein complexes. Proc. Natl. Acad. Sci. U. S. A *97*, 2858-2863.

Cupers,P., Jadhav,A.P., and Kirchhausen,T. (1998). Assembly of clathrin coats disrupts the association between Eps15 and AP-2 adaptors. J Biol. Chem. *273*, 1847-1850.

De Vries,A.C. and Kuipers,E.J. (2007). Review article: Helicobacter pylori eradication for the prevention of gastric cancer. Aliment. Pharmacol. Ther. *26 Suppl 2:25-35.*, 25-35.

de Thé G. (2007). Microbial genomes to write our history. J Infect. Dis. *196*, 499-501.

Delon,I. and Brown,N.H. (2007). Integrins and the actin cytoskeleton. Curr. Opin. Cell Biol. *19*, 43-50.

Dersch,P. and Isberg,R.R. (1999). A region of the Yersinia pseudotuberculosis invasin protein enhances integrin-mediated uptake into mammalian cells and promotes self-association. EMBO J. *18*, 1199-1213.

Dubnau,D. (1999). DNA uptake in bacteria. Annu. Rev. Microbiol *53*, 217-244.

Ehrlich,M., Boll,W., Van,O.A., Hariharan,R., Chandran,K., Nibert,M.L., and Kirchhausen,T. (2004). Endocytosis by random initiation and stabilization of clathrin-coated pits. Cell *118*, 591-605.

el,T.Y. and Skurnik,M. (2001). YadA, the multifaceted Yersinia adhesin. Int J Med Microbiol *291*, 209-218.

Engstrand,L. (2001). Helicobacter in water and waterborne routes of transmission. Symp. Ser. Soc Appl. Microbiol. 80S-84S.

Falush,D., Wirth,T., Linz,B., Pritchard,J.K., Stephens,M., Kidd,M., Blaser,M.J., Graham,D.Y., Vacher,S., Perez-Perez,G.I., Yamaoka,Y., Megraud,F., Otto,K., Reichard,U., Katzowitsch,E., Wang,X., Achtman,M., and Suerbaum,S. (2003). Traces of human migrations in Helicobacter pylori populations. Science. *299*, 1582-1585.

Fassler,R., Pfaff,M., Murphy,J., Noegel,A.A., Johansson,S., Timpl,R., and Albrecht,R. (1995). Lack of beta 1 integrin gene in embryonic stem cells affects morphology, adhesion, and migration but not integration into the inner cell mass of blastocysts. J Cell Biol. *128*, 979-988.

Favre,A., Giunta,M., Gu,Y., Corte,G., and Grossi,C.E. (1992). Localization of a novel integrin of the beta 1 subfamily in human tissues. J Histochem. Cytochem. *40*, 1291-1298.

Fechheimer,M. and Zigmond,S.H. (1983). Changes in cytoskeletal proteins of polymorphonuclear leukocytes induced by chemotactic peptides. Cell Motil. *3*, 349-361.

Ferrero,R.L., Ave,P., Ndiaye,D., Bambou,J.C., Huerre,M.R., Philpott,D.J., and Memet,S. (2008). NF-kappaB activation during acute Helicobacter pylori infection in mice. Infect. Immun. *76*, 551-561.

Ferrero,R.L., Cussac,V., Courcoux,P., and Labigne,A. (1992). Construction of isogenic urease-negative mutants of Helicobacter pylori by allelic exchange. J Bacteriol. *174*, 4212-4217.

Fiorentini,C., Fabbri,A., Flatau,G., Donelli,G., Matarrese,P., Lemichez,E., Falzano,L., and Boquet,P. (1997). Escherichia coli cytotoxic necrotizing factor 1 (CNF1), a toxin that activates the Rho GTPase. J Biol. Chem. *272*, 19532-19537.

Fischer,W., Haas,R., and Odenbreit,S. (2002). Type IV secretion systems in pathogenic bacteria. Int. J Med Microbiol *292*, 159-168.

Fischer,W., Puls,J., Buhrdorf,R., Gebert,B., Odenbreit,S., and Haas,R. (2001). Systematic mutagenesis of the Helicobacter pylori cag pathogenicity island: essential genes for CagA translocation in host cells and induction of interleukin-8. Mol. Microbiol. *42*, 1337-1348.

Fowler,T., Wann,E.R., Joh,D., Johansson,S., Foster,T.J., and Hook,M. (2000). Cellular invasion by Staphylococcus aureus involves a fibronectin bridge between the bacterial fibronectin-binding MSCRAMMs and host cell beta1 integrins. Eur. J. Cell Biol. *79*, 672-679.

Frankel,G., Lider,O., Hershkoviz,R., Mould,A.P., Kachalsky,S.G., Candy,D.C., Cahalon,L., Humphries,M.J., and Dougan,G. (1996). The cell-binding domain of intimin from enteropathogenic Escherichia coli binds to beta1 integrins. J Biol. Chem. *271*, 20359-20364.

Friedl,P., Noble,P.B., and Zanker,K.S. (1993). Lymphocyte locomotion in three-dimensional collagen gels. Comparison of three quantitative methods for analysing cell trajectories. J Immunol. Methods *165*, 157-165.

Futrelle,R.P. (1982). Dictyostelium chemotactic response to spatial and temporal gradients. Theories of the limits of chemotactic sensitivity and of pseudochemotaxis. J Cell Biochem. *18*, 197-212.

Gailit,J. and Ruoslahti,E. (1988). Regulation of the fibronectin receptor affinity by divalent cations. J Biol. Chem. *263*, 12927-12932.

Galan,J.E. (2001). Salmonella interactions with host cells: type III secretion at work. Annu. Rev. Cell Dev. Biol. *17*, 53-86.

Gao,J.X. and Issekutz,A.C. (1995). Polymorphonuclear leucocyte migration through human dermal fibroblast monolayers is dependent on both beta 2-integrin (CD11/CD18) and beta 1-integrin (CD29) mechanisms. Immunology *85*, 485-494.

Gao,J.X., Wilkins,J., and Issekutz,A.C. (1995). Migration of human polymorphonuclear leukocytes through a synovial fibroblast barrier is mediated by both beta 2 (CD11/CD18) integrins and the beta 1 (CD29) integrins VLA-5 and VLA-6. Cell Immunol. *163*, 178-186.

Gebert,B., Fischer,W., Weiss,E., Hoffmann,R., and Haas,R. (2003). Helicobacter pylori vacuolating cytotoxin inhibits T lymphocyte activation. Science *301*, 1099-1102.

Ginsberg,M.H., Yaspan,B., Forsyth,J., Ulmer,T.S., Campbell,I.D., and Slepak,M. (2001). A membrane-distal segment of the integrin alpha IIb cytoplasmic domain regulates integrin activation. J Biol. Chem. *276*, 22514-22521.

Gomez-Mouton,C., Abad,J.L., Mira,E., Lacalle,R.A., Gallardo,E., Jimenez-Baranda,S., Illa,I., Bernad,A., Manes,S., and Martinez,A. (2001). Segregation of leading-edge and uropod components into specific lipid rafts during T cell polarization. Proc. Natl. Acad. Sci. U. S. A *98*, 9642-9647.

Gonzalez,A.M., Gonzales,M., Herron,G.S., Nagavarapu,U., Hopkinson,S.B., Tsuruta,D., and Jones,J.C. (2002). Complex interactions between the laminin alpha 4 subunit and integrins regulate endothelial cell behavior in vitro and angiogenesis in vivo. Proc. Natl. Acad. Sci. U. S. A *99*, 16075-16080.

Graham,E.A. (1922). SURGICAL TREATMENT OF SYPHILIS OF THE STOMACH. Ann. Surg. *76*, 449-456.

Greenlee,R.T., Murray,T., Bolden,S., and Wingo,P.A. (2000). Cancer statistics, 2000. CA Cancer J Clin. *50*, 7-33.

Hall,D.E., Reichardt,L.F., Crowley,E., Holley,B., Moezzi,H., Sonnenberg,A., and Damsky,C.H. (1990). The alpha 1/beta 1 and alpha 6/beta 1 integrin heterodimers mediate cell attachment to distinct sites on laminin. J Cell Biol. *110*, 2175-2184.

Hamzaoui,N., Kerneis,S., Caliot,E., and Pringault,E. (2004). Expression and distribution of beta1 integrins in in vitro-induced M cells: implications for Yersinia adhesion to Peyer's patch epithelium. Cell Microbiol *6*, 817-828.

Hatakeyama,M. (2006). The role of Helicobacter pylori CagA in gastric carcinogenesis. Int J Hematol. *84*, 301-308.

Hauert,A.B., Martinelli,S., Marone,C., and Niggli,V. (2002). Differentiated HL-60 cells are a valid model system for the analysis of human neutrophil migration and chemotaxis. Int. J. Biochem. Cell Biol. *34*, 838-854.

Heesemann,J. and Aepfelbacher,M. (2001). The fight between cells and bacteria: modulation of eukaryotic signalling by bacterial pathogens. Int J Med Microbiol *291*, 241-242.

Heit,B., Tavener,S., Raharjo,E., and Kubes,P. (2002). An intracellular signaling hierarchy determines direction of migration in opposing chemotactic gradients. J. Cell Biol. *159*, 91-102.

Helle,F. and Dubuisson,J. (2008). Hepatitis C virus entry into host cells. Cell Mol. Life Sci. *65*, 100-112.

Higashi,H., Tsutsumi,R., Fujita,A., Yamazaki,S., Asaka,M., Azuma,T., and Hatakeyama,M. (2002). Biological activity of the Helicobacter pylori virulence factor CagA is determined by variation in the tyrosine phosphorylation sites. Proc. Natl. Acad. Sci. U. S. A *99*, 14428-14433.

Higashi,H., Yokoyama,K., Fujii,Y., Ren,S., Yuasa,H., Saadat,I., Murata-Kamiya,N., Azuma,T., and Hatakeyama,M. (2005). EPIYA motif is a membrane-targeting signal of Helicobacter pylori virulence factor CagA in mammalian cells. J Biol. Chem. *280*, 23130-23137.

Hoffmann,S., Maschuw,K., Hassan,I., Reckzeh,B., Wunderlich,A., Lingelbach,S., and Zielke,A. (2005). Differential pattern of integrin receptor expression in differentiated and anaplastic thyroid cancer cell lines. Thyroid. *15*, 1011-1020.

Hofreuter,D., Odenbreit,S., and Haas,R. (2001). Natural transformation competence in Helicobacter pylori is mediated by the basic components of a type IV secretion system. Mol. Microbiol. *41*, 379-391.

Hofreuter,D., Odenbreit,S., Henke,G., and Haas,R. (1998). Natural competence for DNA transformation in Helicobacter pylori: identification and genetic characterization of the comB locus. Mol. Microbiol. *28*, 1027-1038.

Holmes,D.S. and Quigley,M. (1981). A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. *114*, 193-197.

Hsia,D.A., Mitra,S.K., Hauck,C.R., Streblow,D.N., Nelson,J.A., Ilic,D., Huang,S., Li,E., Nemerow,G.R., Leng,J., Spencer,K.S., Cheresh,D.A., and Schlaepfer,D.D. (2003). Differential regulation of cell motility and invasion by FAK. J. Cell Biol. *160*, 753-767.

Huttenlocher,A. (2005). Cell polarization mechanisms during directed cell migration. Nat. Cell Biol. *7*, 336-337.

Ilver,D., Arnqvist,A., Ogren,J., Frick,I.M., Kersulyte,D., Incecik,E.T., Berg,D.E., Covacci,A., Engstrand,L., and Boren,T. (1998). Helicobacter pylori adhesin binding fucosylated histo-blood group antigens revealed by retagging. Science *279*, 373-377.

Innocenti,M., Thoreson,A.C., Ferrero,R.L., Stromberg,E., Bolin,I., Eriksson,L., Svennerholm,A.M., and Quiding-Jarbrink,M. (2002). Helicobacter pylori-induced activation of human endothelial cells. Infect. Immun. *70*, 4581-4590.

Isberg,R.R. and Leong,J.M. (1990). Multiple beta 1 chain integrins are receptors for invasin, a protein that promotes bacterial penetration into mammalian cells. Cell *60*, 861-871.

Itoh,K., Okubo,K., Utiyama,H., Hirano,T., Yoshii,J., and Matsubara,K. (1998). Expression profile of active genes in granulocytes. Blood *92*, 1432-1441.

Joseph,I.M., Zavros,Y., Merchant,J.L., and Kirschner,D. (2003). A model for integrative study of human gastric acid secretion. J Appl. Physiol. *94*, 1602-1618.

Kamata,T., Ambo,H., Puzon-McLaughlin,W., Tieu,K.K., Handa,M., Ikeda,Y., and Takada,Y. (2004). Critical cysteine residues for regulation of integrin alphaIIbbeta3 are clustered in the epidermal growth factor domains of the beta3 subunit. Biochem. J *378*, 1079-1082.

Karnholz,A., Hoefler,C., Odenbreit,S., Fischer,W., Hofreuter,D., and Haas,R. (2006). Functional and topological characterization of novel components of the comB DNA transformation competence system in Helicobacter pylori. J Bacteriol. *188*, 882-893.

Kavermann,H., Burns,B.P., Angermuller,K., Odenbreit,S., Fischer,W., Melchers,K., and Haas,R. (2003). Identification and characterization of Helicobacter pylori genes essential for gastric colonization. J. Exp. Med. *197*, 813-822.

Kersulyte,D., Velapatino,B., Mukhopadhyay,A.K., Cahuayme,L., Bussalleu,A., Combe,J., Gilman,R.H., and Berg,D.E. (2003). Cluster of type IV secretion genes in Helicobacter pylori's plasticity zone. J Bacteriol *185*, 3764-3772.

Kihira,K., Satoh,K., Saifuku,K., Taniguchi,Y., Takimoto,T., Yoshida,Y., Ishino,Y., Yamamoto,H., and Kimura,K. (1996). Endoscopic topical therapy for the treatment of Helicobacter pylori infection. J Gastroenterol. *31 Suppl 9:66-8.*, 66-68.

Kingsley,R.A., Abi,G.D., Puebla-Osorio,N., Keestra,A.M., Berghman,L., and Baumler,A.J. (2004). Fibronectin binding to the Salmonella enterica serotype Typhimurium ShdA autotransporter protein is inhibited by a monoclonal antibody recognizing the A3 repeat. J Bacteriol. *186*, 4931-4939.

Kirchhausen,T. (1999). Adaptors for clathrin-mediated traffic. Annu. Rev. Cell Dev. Biol. *15:705-32.*, 705-732.

Krysko,A.A., Chugunov,B.M., Malovichko,O.L., Andronati,S.A., Kabanova,T.A., Karaseva,T.L., and Kiriyak,A.V. (2004). Novel fibrinogen receptor antagonists. RGDF mimetics, derivatives of 4-(isoindoline-5-yl)amino-4-oxobutyric acid. Bioorg. Med Chem. Lett. *14*, 5533-5535.

Kutter,S., Buhrdorf,R., Haas,J., Schneider-Brachert,W., Haas,R., and Fischer,W. (2008). Protein subassemblies of the Helicobacter pylori Cag type IV secretion system revealed by localization and interaction studies. J Bacteriol. *190*, 2161-2171.

Kuwada,S.K. and Li,X. (2000). Integrin alpha5/beta1 mediates fibronectin-dependent epithelial cell proliferation through epidermal growth factor receptor activation. Mol. Biol. Cell *11*, 2485-2496.

Kwok,T., Zabler,D., Urman,S., Rohde,M., Hartig,R., Wessler,S., Misselwitz,R., Berger,J., Sewald,N., Konig,W., and Backert,S. (2007). Helicobacter exploits integrin for type IV secretion and kinase activation. Nature. *449*, 862-866.

Lammermann,T., Bader,B.L., Monkley,S.J., Worbs,T., Wedlich-Soldner,R., Hirsch,K., Keller,M., Forster,R., Critchley,D.R., Fassler,R., and Sixt,M. (2008). Rapid leukocyte migration by integrin-independent flowing and squeezing. Nature. *453*, 51-55.

Lee,J.O., Bankston,L.A., Arnaout,M.A., and Liddington,R.C. (1995). Two conformations of the integrin A-domain (I-domain): a pathway for activation? Structure. *3*, 1333-1340.

Legate,K.R., Montanez,E., Kudlacek,O., and Fassler,R. (2006). ILK, PINCH and parvin: the tIPP of integrin signalling. Nat. Rev. Mol. Cell Biol. *7*, 20-31.

Leitinger,B. and Hogg,N. (2002). The involvement of lipid rafts in the regulation of integrin function. J Cell Sci. *115*, 963-972.

Lenter,M., Uhlig,H., Hamann,A., Jeno,P., Imhof,B., and Vestweber,D. (1993). A monoclonal antibody against an activation epitope on mouse integrin chain beta 1 blocks adhesion of lymphocytes to the endothelial integrin alpha 6 beta 1. Proc. Natl. Acad. Sci. U. S. A *90*, 9051-9055.

Leong,J.M., Morrissey,P.E., Marra,A., and Isberg,R.R. (1995). An aspartate residue of the Yersinia pseudotuberculosis invasin protein that is critical for integrin binding. EMBO J *14*, 422-431.

Linz,B., Balloux,F., Moodley,Y., Manica,A., Liu,H., Roumagnac,P., Falush,D., Stamer,C., Prugnolle,F., van der Merwe,S.W., Yamaoka,Y., Graham,D.Y., Perez-Trallero,E., Wadstrom,T., Suerbaum,S., and Achtman,M. (2007). An African origin for the intimate association between humans and Helicobacter pylori. Nature. *445*, 915-918.

Liu,G., McDaniel,T.K., Falkow,S., and Karlin,S. (1999). Sequence anomalies in the Cag7 gene of the Helicobacter pylori pathogenicity island. Proc. Natl. Acad. Sci. U. S. A *96*, 7011-7016.

Liu,S., Calderwood,D.A., and Ginsberg,M.H. (2000). Integrin cytoplasmic domain-binding proteins. J. Cell Sci. *113 (Pt 20)*, 3563-3571.

Lock,J.G., Wehrle-Haller,B., and Stromblad,S. (2008). Cell-matrix adhesion complexes: master control machinery of cell migration. Semin. Cancer Biol. *18*, 65-76.

Lu,C., Oxvig,C., and Springer,T.A. (1998). The structure of the beta-propeller domain and C-terminal region of the integrin alphaM subunit. Dependence on beta subunit association and prediction of domains. J Biol. Chem. *273*, 15138-15147.

Macia,E., Ehrlich,M., Massol,R., Boucrot,E., Brunner,C., and Kirchhausen,T. (2006). Dynasore, a cell-permeable inhibitor of dynamin. Dev. Cell. *10*, 839-850.

MADDING,G.F., BAER,L.S., and KENNEDY,P.A. (1964). GASTRIC SYPHILIS: A CASE REPORT. Ann. Surg. *159:271-4.*, 271-274.

Mahdavi,J., Sonden,B., Hurtig,M., Olfat,F.O., Forsberg,L., Roche,N., Angstrom,J., Larsson,T., Teneberg,S., Karlsson,K.A., Altraja,S., Wadstrom,T., Kersulyte,D., Berg,D.E., Dubois,A., Petersson,C., Magnusson,K.E., Norberg,T., Lindh,F., Lundskog,B.B., Arnqvist,A., Hammarstrom,L., and Boren,T. (2002). Helicobacter pylori SabA adhesin in persistent infection and chronic inflammation. Science. *297*, 573-578.

Malecki,J., Wiedlocha,A., Wesche,J., and Olsnes,S. (2002). Vesicle transmembrane potential is required for translocation to the cytosol of externally added FGF-1. EMBO J. *21*, 4480-4490.

Marlink,K.L., Bacon,K.D., Sheppard,B.C., Ashktorab,H., Smoot,D.T., Cover,T.L., Deveney,C.W., and Rutten,M.J. (2003). Effects of Helicobacter pylori on intracellular Ca2+

signaling in normal human gastric mucous epithelial cells. Am. J. Physiol Gastrointest. Liver Physiol. *285*, G163-G176.

Marshall,B.J., Armstrong,J.A., McGechie,D.B., and Glancy,R.J. (1985). Attempt to fulfil Koch's postulates for pyloric Campylobacter. Med. J. Aust. *142*, 436-439.

Marshall,B.J. and Warren,J.R. (1984). Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet *1*, 1311-1315.

McColl,K.E., Watabe,H., and Derakhshan,M.H. (2008). Role of gastric atrophy in mediating negative association between Helicobacter pylori infection and reflux oesophagitis, Barrett's oesophagus and oesophageal adenocarcinoma. Gut. *57*, 721-723.

McDowall,A., Inwald,D., Leitinger,B., Jones,A., Liesner,R., Klein,N., and Hogg,N. (2003). A novel form of integrin dysfunction involving beta1, beta2, and beta3 integrins. J Clin. Invest *111*, 51-60.

Megraud,F. (2007). Detection of Helicobacter pylori and its sensitivity to antibiotics. A step forward in the use of molecular methods. Gastroenterol. Clin. Biol. *31*, 790-791.

Meier,O. and Greber,U.F. (2004). Adenovirus endocytosis. J Gene Med *6 Suppl 1*, S152-S163.

Meili,R. and Firtel,R.A. (2003). Two poles and a compass. Cell *114*, 153-156.

Miaczynska,M. and Stenmark,H. (2008). Mechanisms and functions of endocytosis. J Cell Biol. *180*, 7-11.

Moese,S., Selbach,M., Kwok,T., Brinkmann,V., Konig,W., Meyer,T.F., and Backert,S. (2004). Helicobacter pylori induces AGS cell motility and elongation via independent signaling pathways. Infect. Immun. *72*, 3646-3649.

Montecucco,C., De Bernard,M., Papini,E., and Zoratti,M. (2001). Helicobacter pylori vacuolating cytotoxin: cell intoxication and anion-specific channel activity. Curr Top Microbiol Immunol *257*, 113-129.

Mould,A.P., Akiyama,S.K., and Humphries,M.J. (1995). Regulation of integrin alpha 5 beta 1-fibronectin interactions by divalent cations. Evidence for distinct classes of binding sites for Mn2+, Mg2+, and Ca2+. J. Biol. Chem. *270*, 26270-26277.

Mould,A.P., Askari,J.A., Aota,S., Yamada,K.M., Irie,A., Takada,Y., Mardon,H.J., and Humphries,M.J. (1997). Defining the topology of integrin alpha5beta1-fibronectin interactions using inhibitory anti-alpha5 and anti-beta1 monoclonal antibodies. Evidence that the synergy sequence of fibronectin is recognized by the amino-terminal repeats of the alpha5 subunit. J. Biol. Chem. *272*, 17283-17292.

Mould,A.P., Askari,J.A., Barton,S., Kline,A.D., McEwan,P.A., Craig,S.E., and Humphries,M.J. (2002). Integrin activation involves a conformational change in the alpha 1 helix of the beta subunit A-domain. J Biol. Chem. *277*, 19800-19805.

Mould,A.P., Travis,M.A., Barton,S.J., Hamilton,J.A., Askari,J.A., Craig,S.E., Macdonald,P.R., Kammerer,R.A., Buckley,P.A., and Humphries,M.J. (2005). Evidence that

monoclonal antibodies directed against the integrin beta subunit plexin/semaphorin/integrin domain stimulate function by inducing receptor extension. J Biol. Chem. *280*, 4238-4246.

Mousavi,S.A., Malerod,L., Berg,T., and Kjeken,R. (2004). Clathrin-dependent endocytosis. Biochem. J. *377*, 1-16.

Mueller,C.A., Broz,P., and Cornelis,G.R. (2008). The type III secretion system tip complex and translocon. Mol. Microbiol *68*, 1085-1095.

Mukherjee,S. and Maxfield,F.R. (2004). Membrane domains. Annu. Rev. Cell Dev. Biol. *20:839-66.*, 839-866.

Muller,W.A. (2003). Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response. Trends Immunol. *24*, 327-334.

Murata-Kamiya,N., Kurashima,Y., Teishikata,Y., Yamahashi,Y., Saito,Y., Higashi,H., Aburatani,H., Akiyama,T., Peek,R.M., Jr., Azuma,T., and Hatakeyama,M. (2007). Helicobacter pylori CagA interacts with E-cadherin and deregulates the beta-catenin signal that promotes intestinal transdifferentiation in gastric epithelial cells. Oncogene. *26*, 4617-4626.

Namavar,F., Sparrius,M., Veerman,E.C., Appelmelk,B.J., and Vandenbroucke-Grauls,C.M. (1998). Neutrophil-activating protein mediates adhesion of Helicobacter pylori to sulfated carbohydrates on high-molecular-weight salivary mucin. Infect. Immun. *66*, 444-447.

Nankoe,S.R. and Sever,S. (2006). Dynasore puts a new spin on dynamin: a surprising dual role during vesicle formation. Trends Cell Biol. *16*, 607-609.

Nhieu,G.T. and Sansonetti,P.J. (1999). Mechanism of Shigella entry into epithelial cells. Curr. Opin. Microbiol *2*, 51-55.

Ni,H., Li,A., Simonsen,N., and Wilkins,J.A. (1998). Integrin activation by dithiothreitol or Mn2+ induces a ligand-occupied conformation and exposure of a novel NH2-terminal regulatory site on the beta1 integrin chain. J Biol. Chem. *273*, 7981-7987.

Ni,H. and Wilkins,J.A. (1998). Localisation of a novel adhesion blocking epitope on the human beta 1 integrin chain. Cell Adhes. Commun. *5*, 257-271.

Nozawa,Y., Nishihara,K., Akizawa,Y., Orimoto,N., Nakano,M., Uji,T., Ajioka,H., Kanda,A., Matsuura,N., and Kiniwa,M. (2004). Protein kinase C activation by Helicobacter pylori in human gastric epithelial cells limits interleukin-8 production through suppression of extracellular signal-regulated kinase. J. Pharmacol. Sci. *94*, 233-239.

Odenbreit,S. and Haas,R. (2002). Helicobacter pylori: impact of gene transfer and the role of the cag pathogenicity island for host adaptation and virulence. Curr Top Microbiol Immunol *264*, 1-22.

Odenbreit,S., Puls,J., Sedlmaier,B., Gerland,E., Fischer,W., and Haas,R. (2000). Translocation of Helicobacter pylori CagA into gastric epithelial cells by type IV secretion. Science *287*, 1497-1500.

Odenbreit,S., Till,M., Hofreuter,D., Faller,G., and Haas,R. (1999). Genetic and functional characterization of the alpAB gene locus essential for the adhesion of Helicobacter pylori to human gastric tissue. Mol. Microbiol. *31*, 1537-1548.

Okubo,K., Itoh,K., Fukushima,A., Yoshii,J., and Matsubara,K. (1995). Monitoring cell physiology by expression profiles and discovering cell type-specific genes by compiled expression profiles. Genomics *30*, 178-186.

Owen,D.J., Collins,B.M., and Evans,P.R. (2004). Adaptors for clathrin coats: structure and function. Annu. Rev. Cell Dev. Biol. *20:153-91.*, 153-191.

Owen,R.J. (1998). Helicobacter--species classification and identification. Br. Med Bull. *54*, 17-30.

Oxvig,C. and Springer,T.A. (1998). Experimental support for a beta-propeller domain in integrin alpha-subunits and a calcium binding site on its lower surface. Proc. Natl. Acad. Sci. U. S. A *95*, 4870-4875.

Pattis,I., Weiss,E., Laugks,R., Haas,R., and Fischer,W. (2007). The Helicobacter pylori CagF protein is a type IV secretion chaperone-like molecule that binds close to the C-terminal secretion signal of the CagA effector protein. Microbiology *153*, 2896-2909.

Pelicic,V., Reyrat,J.M., Sartori,L., Pagliaccia,C., Rappuoli,R., Telford,J.L., Montecucco,C., and Papini,E. (1999). Helicobacter pylori VacA cytotoxin associated with the bacteria increases epithelial permeability independently of its vacuolating activity. Microbiology *145 (Pt 8)*, 2043-2050.

Phonphok,Y. and Rosenthal,K.S. (1991). Stabilization of clathrin coated vesicles by amantadine, tromantadine and other hydrophobic amines. FEBS Lett. *281*, 188-190.

Poppe,M., Feller,S.M., Romer,G., and Wessler,S. (2007). Phosphorylation of Helicobacter pylori CagA by c-Abl leads to cell motility. Oncogene. *26*, 3462-3472.

Pozzi,A. and Zent,R. (2003). integrins: sensors of extracellular matrix and modulators of cell function. Nephron Exp. Nephrol. *94*, e77-e84.

Puertollano,R. (2004). Clathrin-mediated transport: assembly required. Workshop on Molecular Mechanisms of Vesicle Selectivity. EMBO Rep. *5*, 942-946.

Rappoport,J.Z., Kemal,S., Benmerah,A., and Simon,S.M. (2006). Dynamics of clathrin and adaptor proteins during endocytosis. Am. J Physiol Cell Physiol. *291*, C1072-C1081.

Rappoport,J.Z., Simon,S.M., and Benmerah,A. (2004). Understanding living clathrin-coated pits. Traffic. *5*, 327-337.

Rappoport,J.Z., Taha,B.W., and Simon,S.M. (2003). Movement of plasma-membrane-associated clathrin spots along the microtubule cytoskeleton. Traffic. *4*, 460-467.

Retta,S.F., Balzac,F., Ferraris,P., Belkin,A.M., Fassler,R., Humphries,M.J., De,L.G., Silengo,L., and Tarone,G. (1998). beta1-integrin cytoplasmic subdomains involved in dominant negative function. Mol. Biol. Cell. *9*, 715-731.

Robbins,S.M., Quintrell,N.A., and Bishop,J.M. (1995). Myristoylation and differential palmitoylation of the HCK protein-tyrosine kinases govern their attachment to membranes and association with caveolae. Mol. Cell Biol. *15*, 3507-3515.

Rocco,A. and Nardone,G. (2007). Diet, H pylori infection and gastric cancer: evidence and controversies. World J Gastroenterol. *13*, 2901-2912.

Rodal,S.K., Skretting,G., Garred,O., Vilhardt,F., van,D.B., and Sandvig,K. (1999). Extraction of cholesterol with methyl-beta-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles. Mol. Biol. Cell. *10*, 961-974.

Rohde,M., Puls,J., Buhrdorf,R., Fischer,W., and Haas,R. (2003). A novel sheathed surface organelle of the Helicobacter pylori cag type IV secretion system. Mol. Microbiol. *49*, 219-234.

Roux,A., Uyhazi,K., Frost,A., and De,C.P. (2006). GTP-dependent twisting of dynamin implicates constriction and tension in membrane fission. Nature *441*, 528-531.

Satin,B., Del Giudice,G., Della,B., V, Dusi,S., Laudanna,C., Tonello,F., Kelleher,D., Rappuoli,R., Montecucco,C., and Rossi,F. (2000). The neutrophil-activating protein (HP-NAP) of Helicobacter pylori is a protective antigen and a major virulence factor. J. Exp. Med. *191*, 1467-1476.

Schreiber,S., Bucker,R., Groll,C., zevedo-Vethacke,M., Garten,D., Scheid,P., Friedrich,S., Gatermann,S., Josenhans,C., and Suerbaum,S. (2005). Rapid loss of motility of Helicobacter pylori in the gastric lumen in vivo. Infect. Immun. *73*, 1584-1589.

Schreiber,S., Konradt,M., Groll,C., Scheid,P., Hanauer,G., Werling,H.O., Josenhans,C., and Suerbaum,S. (2004). The spatial orientation of Helicobacter pylori in the gastric mucus. Proc. Natl. Acad. Sci. U. S. A. *101*, 5024-5029.

Schulte,R., Zumbihl,R., Kampik,D., Fauconnier,A., and Autenrieth,I.B. (1998). Wortmannin blocks Yersinia invasin-triggered internalization, but not interleukin-8 production by epithelial cells. Med Microbiol Immunol. *187*, 53-60.

Schwartz,J.T. and Allen,L.A. (2006). Role of urease in megasome formation and Helicobacter pylori survival in macrophages. J Leukoc. Biol. *79*, 1214-1225.

Schweighoffer,T., Luce,G.E., Tanaka,Y., and Shaw,S. (1994). Differential expression of integrins alpha 6 and alpha 4 determines pathways in human peripheral CD4+ T cell differentiation. Cell Adhes. Commun. *2*, 403-415.

Sczekan,M.M. and Juliano,R.L. (1990). Protease resistance of the beta subunit of the hamster fibronectin receptor. Evidence for differential cleavage of membrane-bound and soluble receptor. Eur. J Biochem. *189*, 83-87.

Segal,E.D., Falkow,S., and Tompkins,L.S. (1996). Helicobacter pylori attachment to gastric cells induces cytoskeletal rearrangements and tyrosine phosphorylation of host cell proteins. Proc. Natl. Acad. Sci. U. S. A *93*, 1259-1264.

Selbach,M., Moese,S., Hauck,C.R., Meyer,T.F., and Backert,S. (2002). Src is the kinase of the Helicobacter pylori CagA protein in vitro and in vivo. J Biol. Chem. *277*, 6775-6778.

Selbach,M., Moese,S., Hurwitz,R., Hauck,C.R., Meyer,T.F., and Backert,S. (2003). The Helicobacter pylori CagA protein induces cortactin dephosphorylation and actin rearrangement by c-Src inactivation. EMBO J. *22*, 515-528.

Sewald,X., Gebert-Vogl,B., Prassl,S., Barwig,I., Weiss,E., Fabbri,M., Osicka,R., Schiemann,M., Busch,D.H., Semmrich,M., Holzmann,B., Sebo,P., and Haas,R. (2008). Integrin subunit CD18 Is the T-lymphocyte receptor for the Helicobacter pylori vacuolating cytotoxin. Cell Host. Microbe *3*, 20-29.

Sinha,B., Francois,P.P., Nusse,O., Foti,M., Hartford,O.M., Vaudaux,P., Foster,T.J., Lew,D.P., Herrmann,M., and Krause,K.H. (1999). Fibronectin-binding protein acts as Staphylococcus aureus invasin via fibronectin bridging to integrin alpha5beta1. Cell Microbiol. *1*, 101-117.

Sixt,M., Bauer,M., Lammermann,T., and Fassler,R. (2006). Beta1 integrins: zip codes and signaling relay for blood cells. Curr. Opin. Cell Biol. *18*, 482-490.

Smeets,L.C. and Kusters,J.G. (2002). Natural transformation in Helicobacter pylori: DNA transport in an unexpected way. Trends Microbiol. *10*, 159-162.

Smith,J.W. and Cheresh,D.A. (1990). Integrin (alpha v beta 3)-ligand interaction. Identification of a heterodimeric RGD binding site on the vitronectin receptor. J Biol. Chem. *265*, 2168-2172.

Srinivasan,S., Wang,F., Glavas,S., Ott,A., Hofmann,F., Aktories,K., Kalman,D., and Bourne,H.R. (2003). Rac and Cdc42 play distinct roles in regulating PI(3,4,5)P3 and polarity during neutrophil chemotaxis. J. Cell Biol. *160*, 375-385.

Stewart,M.P., McDowall,A., and Hogg,N. (1998). LFA-1-mediated adhesion is regulated by cytoskeletal restraint and by a Ca2+-dependent protease, calpain. J Cell Biol. *140*, 699-707.

Suerbaum,S. and Michetti,P. (2002). Helicobacter pylori infection. N. Engl. J Med. *347*, 1175-1186.

Suzuki,M., Mimuro,H., Suzuki,T., Park,M., Yamamoto,T., and Sasakawa,C. (2005). Interaction of CagA with Crk plays an important role in Helicobacter pylori-induced loss of gastric epithelial cell adhesion. J. Exp. Med. *202*, 1235-1247.

Takada,Y. and Puzon,W. (1993). Identification of a regulatory region of integrin beta 1 subunit using activating and inhibiting antibodies. J Biol. Chem. *268*, 17597-17601.

Takagi,J. and Springer,T.A. (2002). Integrin activation and structural rearrangement. Immunol. Rev. *186*, 141-163.

Tanaka,J., Suzuki,T., Mimuro,H., and Sasakawa,C. (2003). Structural definition on the surface of Helicobacter pylori type IV secretion apparatus. Cell Microbiol. *5*, 395-404.

Teneberg,S., Miller-Podraza,H., Lampert,H.C., Evans,D.J., Jr., Evans,D.G., Danielsson,D., and Karlsson,K.A. (1997). Carbohydrate binding specificity of the neutrophil-activating protein of Helicobacter pylori. J Biol. Chem. *272*, 19067-19071.

Tomb,J.F., White,O., Kerlavage,A.R., Clayton,R.A., Sutton,G.G., Fleischmann,R.D., Ketchum,K.A., Klenk,H.P., Gill,S., Dougherty,B.A., Nelson,K., Quackenbush,J., Zhou,L., Kirkness,E.F., Peterson,S., Loftus,B., Richardson,D., Dodson,R., Khalak,H.G., Glodek,A.,

McKenney,K., Fitzegerald,L.M., Lee,N., Adams,M.D., Hickey,E.K., Berg,D.E., Gocayne,J.D., Utterback,T.R., Peterson,J.D., Kelley,J.M., Cotton,M.D., Weidman,J.M., Fujii,C., Bowman,C., Watthey,L., Wallin,E., Hayes,W.S., Borodovsky,M., Karp,P.D., Smith,H.O., Fraser,C.M., and Venter,J.C. (1997). The complete genome sequence of the gastric pathogen Helicobacter pylori. Nature. *388*, 539-547.

Tonello,F., Dundon,W.G., Satin,B., Molinari,M., Tognon,G., Grandi,G., Del,G.G., Rappuoli,R., and Montecucco,C. (1999). The Helicobacter pylori neutrophil-activating protein is an iron-binding protein with dodecameric structure. Mol. Microbiol. *34*, 238-246.

Travis,M.A., Humphries,J.D., and Humphries,M.J. (2003). An unraveling tale of how integrins are activated from within. Trends Pharmacol. Sci. *24*, 192-197.

Trejo,J. (2003). Protease-activated receptors: new concepts in regulation of G protein-coupled receptor signaling and trafficking. J. Pharmacol. Exp. Ther. *307*, 437-442.

Tsutsumi,R., Takahashi,A., Azuma,T., Higashi,H., and Hatakeyama,M. (2006). Focal adhesion kinase is a substrate and downstream effector of SHP-2 complexed with Helicobacter pylori CagA. Mol. Cell Biol. *26*, 261-276.

Unemo,M., spholm-Hurtig,M., Ilver,D., Bergstrom,J., Boren,T., Danielsson,D., and Teneberg,S. (2005). The sialic acid binding SabA adhesin of Helicobacter pylori is essential for nonopsonic activation of human neutrophils. J Biol. Chem. *280*, 15390-15397.

Van Haastert,P.J. and Devreotes,P.N. (2004). Chemotaxis: signalling the way forward. Nat. Rev. Mol. Cell Biol. *5*, 626-634.

Van Nhieu,G.T. and Isberg,R.R. (1991). The Yersinia pseudotuberculosis invasin protein and human fibronectin bind to mutually exclusive sites on the alpha 5 beta 1 integrin receptor. J Biol. Chem. *266*, 24367-24375.

Viala,J., Chaput,C., Boneca,I.G., Cardona,A., Girardin,S.E., Moran,A.P., Athman,R., Memet,S., Huerre,M.R., Coyle,A.J., DiStefano,P.S., Sansonetti,P.J., Labigne,A., Bertin,J., Philpott,D.J., and Ferrero,R.L. (2004). Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. Nat. Immunol. *5*, 1166-1174.

Vitte,J., Benoliel,A.M., Eymeric,P., Bongrand,P., and Pierres,A. (2004). Beta-1 integrin-mediated adhesion may be initiated by multiple incomplete bonds, thus accounting for the functional importance of receptor clustering. Biophys. J *86*, 4059-4074.

Wang,B., Yurecko,R.S., Dedhar,S., and Cleary,P.P. (2006). Integrin-linked kinase is an essential link between integrins and uptake of bacterial pathogens by epithelial cells. Cell Microbiol. *8*, 257-266.

Wegener,K.L., Partridge,A.W., Han,J., Pickford,A.R., Liddington,R.C., Ginsberg,M.H., and Campbell,I.D. (2007). Structural basis of integrin activation by talin. Cell. *128*, 171-182.

Wesche,J., Malecki,J., Wiedlocha,A., Skjerpen,C.S., Claus,P., and Olsnes,S. (2006). FGF-1 and FGF-2 require the cytosolic chaperone Hsp90 for translocation into the cytosol and the cell nucleus. J Biol. Chem. *281*, 11405-11412.

Wilde,C., Genth,H., Aktories,K., and Just,I. (2000). Recognition of RhoA by Clostridium botulinum C3 exoenzyme. J Biol. Chem. *275*, 16478-16483.

Wilkins,J.A., Li,A., Ni,H., Stupack,D.G., and Shen,C. (1996). Control of beta1 integrin function. Localization of stimulatory epitopes. J Biol. Chem. *271*, 3046-3051.

Wilkins,J.A., Stupack,D., Stewart,S., and Caixia,S. (1991). Beta 1 integrin-mediated lymphocyte adherence to extracellular matrix is enhanced by phorbol ester treatment. Eur. J Immunol. *21*, 517-522.

Wu,X., Zhao,X., Baylor,L., Kaushal,S., Eisenberg,E., and Greene,L.E. (2001). Clathrin exchange during clathrin-mediated endocytosis. J Cell Biol. *155*, 291-300.

Xiong,J.P., Stehle,T., Diefenbach,B., Zhang,R., Dunker,R., Scott,D.L., Joachimiak,A., Goodman,S.L., and Arnaout,M.A. (2001). Crystal structure of the extracellular segment of integrin alpha Vbeta3. Science *294*, 339-345.

Xu,J., Wang,F., Van Keymeulen,A., Herzmark,P., Straight,A., Kelly,K., Takuwa,Y., Sugimoto,N., Mitchison,T., and Bourne,H.R. (2003). Divergent signals and cytoskeletal assemblies regulate self-organizing polarity in neutrophils. Cell *114*, 201-214.

Yanagida,M., Nakayama,H., Yoshizaki,F., Fujimura,T., Takamori,K., Ogawa,H., and Iwabuchi,K. (2007). Proteomic analysis of plasma membrane lipid rafts of HL-60 cells. Proteomics. *7*, 2398-2409.

Yarar,D., Waterman-Storer,C.M., and Schmid,S.L. (2005). A dynamic actin cytoskeleton functions at multiple stages of clathrin-mediated endocytosis. Mol. Biol. Cell. *16*, 964-975.

Yeo,M., Park,H.K., Lee,K.M., Lee,K.J., Kim,J.H., Cho,S.W., and Hahm,K.B. (2004). Blockage of HSP 90 modulates Helicobacter pylori-induced IL-8 productions through the inactivation of transcriptional factors of AP-1 and NF-kappaB. Biochem. Biophys. Res. Commun. *320*, 816-824.

Zhou,D., Chen,L.M., Hernandez,L., Shears,S.B., and Galan,J.E. (2001). A Salmonella inositol polyphosphatase acts in conjunction with other bacterial effectors to promote host cell actin cytoskeleton rearrangements and bacterial internalization. Mol. Microbiol *39*, 248-259.

Zigmond,S.H. (1978). Chemotaxis by polymorphonuclear leukocytes. J Cell Biol. *77*, 269-287.

Zupan,J., Hackworth,C.A., Aguilar,J., Ward,D., and Zambryski,P. (2007). VirB1* promotes T-pilus formation in the vir-Type IV secretion system of Agrobacterium tumefaciens. J Bacteriol. *189*, 6551-6563.

8 Abbreviations

This are the abbreviations used in this work,

Table 8-1 General abbreviations

α	Anti-
βTD	Beta Tail domain
Δ	Delta (deletion)
Aa	Amino acid
Amp	Ampicillin
AP	Alkaline phosphatase
BCIP	Salt of 5-Brom-4-Chlor-3-Indolylphopshate-p-toluidine
BSA	Bovine Serum Albumina
CA	Constitutively Active
Cam	Chloramphenicol
CCP	Clathrin Coated Pits
CCV	Clathrin Coated Vesicles
DN	Dominant Negative
EDTA	Ethylen-diamine-tetraacetate
EGF	Epithelial Growth Factor
EGF (1-4)	EGF-like domains (1 to 4)
EGFR	Epithelial Growth Factor Receptor
ELISA	Enzyme-linked Immunosorbent Assay
EM	Electron microscopy
EPEC	Enteropathogenic *Escherichia coli*
FACS	Fluorescence Activated Cell Sorting
FAK	Focal Adhesion Kinase
FCS	Fetal Calf Serum
FGF1	Fibroblast Growth Factor 1
FITC	Fluorecein-Isothiocyanate
fMLP	Formyl-Methionine-Leucine-Phenylalanine
GFP	Green fluorescent protein
Hsp	Heat shock protein
IgG	Immunoglobulin type G
IL	Interleukine
ILK	Integrin-Linked Kinase
ITGB1	Integrin beta 1
Kan	Kanamycin
KO	Knockout
LB	Luria-Bertani
MBC	Methyl-β-Cyclodextrin
MDC	Mono-dansylcadaverine
MIDAS	Metal Ion-dependent adhesion site
MOI	Multiplicity of Infection
NBT	Nitrotetrazolium blue chloride
NFκB	Nuclear Factor kappa B
PARs	Protease Activated Receptors
PBS	Phosphate-buffered solution
PE	Phycoerithrin

PFA	Paraformalaldehyde
pI	pH of the Isoelectric point
Pox	Peroxidase
PSI	Plexin, Semaphorins and Integrins
RAD	Argininge-Alanine-Asparagine
RGD	Arginine-Glycine-Asparagine
SDS	Sodium Dodecyl Sulfate
siRNA	Small interference RNA
spp	Species
T3SS	Type III Secretion System
T4SS	Type IV Secretion System
TBS	Tris-buffered Solution
TFS3	Type IV secretion 3
Tris	Tris (hydroxymethyl) aminomethane
WHO	World Health Organisation

Table 8-2 Units

°C	Grade Celcius
µ	Micro-, 10^{-6}
A	Ampers
bp	Base pairs
cm^2	Square centimeters
Da	Daltons
g	Standard gravities
g	Grams
h	Hour
k	Kilo, 10^3
l	Liter
m	Milli-, 10^{-3}
M	Molar (mol/l)
n	Nano-, 10^{-9}
rpm	Revolutions per minute
U	Unit
V	Volts

Personal Details

Name: Jimenez Soto, Luisa Fernanda

Date of Birth: 14th August, 1976

Education

04/2003 – 12/2008	PhD Studies. Ludwig Maximilian University, Munich, Germany
09/2001 – 12/2001	Continued studies in Cell and Molecular Biology with Laboratory rotation, Central Connecticut State University – Connecticut, U.S.A
1994 – 1999	University Degree in Microbiology (Microbiologa), Universidad de los Andes
1987 – 1993	Secondary School (Bachillerato), Colegio Santa Clara.
1981 – 1987	Elementary School (Primaria), Colegio Santa Clara.

Other Studies

05/2000 – 11/2000	Emergency Medical Technician – Basic, Norwood Hospital- State of Massachusetts, U.S.A.
05/1999 – 11/1999	English as a Second Language, American Red Cross and Newton Community Education
04/1997 – 07/1997	Management Skills Training, Colombian Red Cross.
08/1996 – 12/1996	Volunteer Emergency Medical Technician, Colombian Red Cross.

Work Experience

04/2003 – 12/2008	Max-von-Pettenkofer Institue, LMU Munich, Germany. Thesis project :Studies on the function of the Type IV Secretion System of Helicobacter pylori with integrin β1.
02/2002 – 02/2003	Yale University. Boyer Center for Molecular Medicine. Department of Microbial Pathogenesis. *Research Assistant II*
01/1998 – 06/1998	Cenicaña, Centro de Investigaciones de la Caña de Azucar de Colombia (Research Center for Sugar Cane). Florida – Valle del Cauca, Colombia URL: http://www.cenicana.org *Research Assistant (Internship)*