Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii
Vol. 22, issue 4, 2012, pp. 483-488
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# CAGA PHOSPHORYLATION IN HELICOBACTER PYLORI INFECTION

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**ABSTRACT.** The ability of CagA, the most studied bacterial virulence factor of Helicobacter pylori to act in both phosphorylated and non phosphorylated form in order to activate multiple signal transduction pathways, promoting disruption of cell-cell contacts, migration and the typical hummingbird phenotype are under inverstigations. The wide variety of cagA effects, a different molecular forms phosphorylated/ non-phosphorylated, lead us to investigate the location of phosphorylation immediately after infection. The cytosolic (cyt) and non cytosolic (noncyt) fractions of host cells were investigated for the ability to phosphorylate cagA, in order to establish the location of components implied in phosphorylation. The results indicate that recombinant FL-cagA can not be phosphorylated only by cytosol or Non-cytosol from MDCK cells. The phosphorylation take place with phosphorylation reaction buffer (ATP, Mg+2, phosphatase inhibitors). That is an evidence for supporting hypothesis that specific kinases located on cell membranes are needed to perform phosphorylation.

**Keywords:** infection, kinase, membrane, virulence factor, cagA, Helicobacter pylori

**Abbreviations**: cyt= cytosolic fractions; noncyt= noncytosolic fractions; Hp, HP= Helicobacter pylori; MDCK = Mardin-Darbey canine kidney cells; rcagA= recombinant cagA, FL= full lenght;WB= western blott; SDS-PAGE= dodecylsulphate polyacrilamide gel electrophoresys; ATP= adenosine triphosphate; Inh= phosphatase inhibitors, pTyr= phosphotyrosin; IB: immunoblott.

## INTRODUCTION

The Gram-negative bacterium Helicobacter pylori is a causative agent of gastritis and peptic ulcer disease in humans. Strains producing the CagA antigen (cagA +) induce strong gastric inflammation and are strongly associated with gastric adenocarcinoma and MALT lymphoma (Oldenbreit, 2000, Yamasaki, 2006).

The Helicobacter pylori cag pathogenicity island encodes a secretory system that translocates CagA into epithelial host cells, where it becomes tyrosine phosphorylated and induces cytoskeletal rearrangements. Phosphorylation of CagA is closely associated with gastric cancer (Argent, 2004).

The ability of CagA, the most studied bacterial virulence factor of Helicobacter pylori to act in both phosphorylated and non phosphorylated form in order to activate multiple signal transduction pathways, promoting disruption of cell-cell contacts, migration and the typical hummingbird phenotype are under investigations (Bouzrac, 2005, Ernst, 2000).

The effects of CagA phosphorylation have been studied with particular intensity. Phosphorylated CagA causes dysregulation of epithelial structure and integrity through its effect on host cell signaling, such as the extracellular signal-regulated kinase (ERK)/ mitogen-activated protein kinase (MAPK) pathway, by interacting with SHP-2, Grb2, Crk/Crk-L, Csk, Met, and ZO-1 (Amieva et al., 2003; Churin et al., 2003; Higashi et al., 2002;Tsutsumi et al., 2003).

Nonphosphorylated CagA also contributes to the development of the Hp-associated gastric illnesses, including gastric cancer.

The CagA tyrosine phosphorylation motifs (TPMs) have been mapped to the Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs present in the C-terminal region of the protein (Zhang, 2005). CagA proteins generally possess two or more EPIYA motifs within the variable region, which proteins isolated from different H. pylori strains. CagA proteins with greater numbers of TPMs become more phosphorylated and lead to enhanced formation of the "hummingbird" phenotype.

The wide variety of cagA effects (Covacci,1993; Chitsazi, 2006; Crew, 2006; Churin, 2003), the different molecular forms phosphorylated/ non-phosphorylated Suzuki, 2009), lead us to investigate the location of phosphorylation immediately after infection. The cytosolic (cyt) and non cytosolic (noncyt) fractions of host cells were investigated for the ability to phosphorylate cagA, in order to establish the location of components implied in phosphorylation.

The biological activity of the phosphorylated form is well established; however, function(s) of the nonphosphorylated form remain elusive (Suzuki, 2009).

Simmilary research was focused on the role of lipid rafts to clustering of cagA and enzymes implied in her phosphorylation.

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## MATERIALS AND METHODS

### *Chemicals*

We have purchased the chemicals from Santa Cruz Biotechnology: polyclonal antibody against CagA after immunization, monoclonal antibody anti c-Jun, anti ERK, anti E-cadherin, anti-beta-Tubulin, Anti pTyr and anti-caveolin.

### *Methods*

1. Cell culture

MDCK (Madin-Darby Canine Kidney Cells) cells were cultured in Eagle medium supplemented with 10% fetal calf serum. Cells were seeds at density 3 x 10 6 on 24x10 cm plates and used at confluence after 3 days.

2. Expression of recombinant CagA

Recombinant CagA, full-length was expressed as fusion protein with GST-Tag E. Coli BL21 protease deficient strain. After the isolation and purification, the GST-CagA 1-1158 (Full length CagA), were obtained in a pure form. To purifiy and eliminate the GST-tag the Glutathione Sepharose High Performance" Amersham Biosciences () was used as descriebed by producer.

3. SDS-PAGE. 10 % was used in experiments, all material were purchased from Bio-Rad Company.

4. Preparation of membranes fractions (non-cyt) and cytosolic fractions (cyt)

MDCK cells were extracted with hipo osmotic buffer. The puffer contains: 50 mM Hepes pH 7.4; 100 mM NaCl; 1% NP-40; 0.5% Laurylmaltoside; 1mM PMSF; 1mM Vanadat; 50 mM NaF; 10 mM sodium pyrophosphate. Infected cells from 3 plates were collected (250 ml Pellet) and added 1 ml puffer. Sample was centrifugated at 1200 rpm and supernatant discharged. Froozen at -80°C for 15 minutes. Ultrasounds 15 minutes on ice. Centrifugation 13000xg, 10 min, 4°C.

5. Testing the efficiency of extraction of proteic fractions using organelle specifically marchers

Proteins extracted were supposed to SDS-PAGE and immunoblotting. Marcher proteins for each subcellular fraction were tested in order to confirm that the obtained extracts represent the expected subcellular fractions: c-Jun indicator for nucleus, ERK-for membranes, beta-tubulin for cytosol, E-cadherin for cytoscheleton, caveolin for nucleus.

The supernatant represents the cyt and pellet represents the non-cyt, membranes fraction.

6. Lipid rafts preparation

Cell lysates after infection of MDCK cells with P1 HP was supossed to centrifugation in sucrose gradient 5%-40% at 200 000 xg for 20 hours at 4 °C. The 10 fraction were collected and analysed SDS-PAGE and WB against cagA and pTyr.

## RESULTS AND DISCUSSIONS

The main question was: Is the cytosol/ Non-cytosol of MDCK cells implied in rCagA phosphorylation?

RcagA was obtained after cloning of cagA gene and expressed in E.Coli strain BCL 21.

**pGEX CagA(1-858)-GST**

Vevtor : pGEX-6P-1

template: *H.pylori* CagA AF202973 (NCTC11637)

**primer**

CagA(1-858)-BamHI 5´: gCggaTCCaTgaCTaaCgaaaC

CagA(1-858)-XhoI 5´: gaCTCgagggCCTCTaTTCC

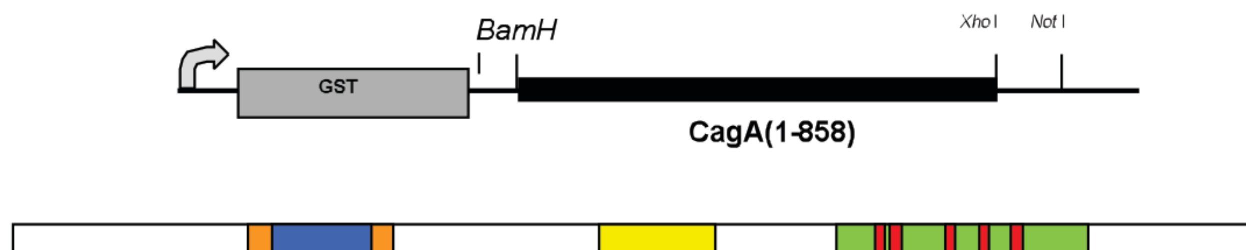


**Fig. 1** Cloning CagA gene cagA. Gene for cagA (rCagA FL), was cloned using vector pGEX, protocol Amersham Bioscience GST

After expression and lysis of bacterial cells (Fig. 1), recombinant full length cagA was isolated and purified as descriebed by manufacturer. r FL-cagA was tested for thei puritiy by SDS-PAGE and Coomassie blue staining. rCagA was obtained in a high purity form as confirmed by SDS-PAGE (Fig. 2).



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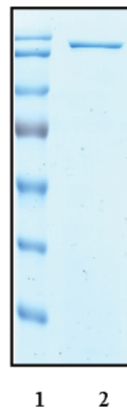


**Fig. 2** Recombinant Fl cagA after 30 minutes ITPG induction. SDS-PAGE to check the purity.
1-molecularmass standards; 2-rcagA

The cytosolic and noncytosolic fraction were tested for their purity using membrane (E-cadherin, c-jun) or cytosol markers (ERK) and confirmed (Fig. 3).

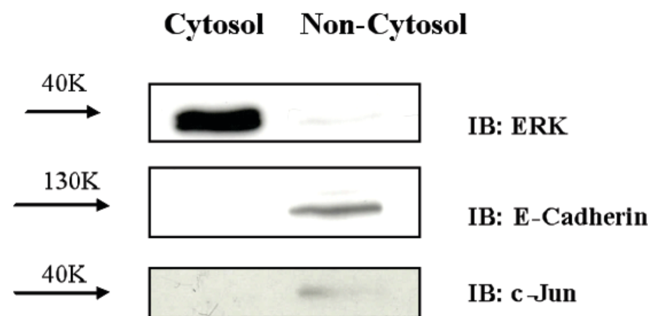


**Fig. 3** Preparation of cytosolic and non-cytosolic fractions. Check the purity offractions.

Analysis of rcagA phosphorylation only in presence of cyt or noncyt shows no implication separately of cyt or noncyt in rcagA phosphorylation (Fig. 4).

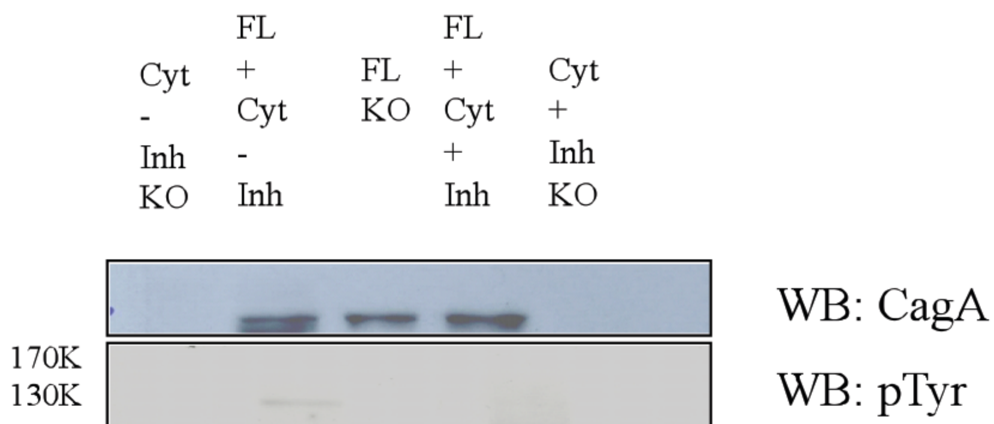


**Fig. 4** Phosphorylation of full lenght rCagA was checked only with cyt respectively non-cyt fractions.
No phosphorylation occuring. Cyt-cytosol, FL-FLrcagA, KO= control; Inh=phosphatase inhibitors


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The phosphorylation was also performed using additionally ATP as substrate and kinases activators ions Mg +2. In this case the positive results appear using noncyt, membranes fraction. As well known, the kinases products are very sensitive to phosphatases, and the confirmation that a kinases reaction occurs can be made only in presence of phosphatase inhibitors (Fig. 5). The noncyt fraction respectively the membranes appear to be implied in phosphorylation of rcagA in vitro in presence of phosphorylation reaction buffer (ATP, Mg+2, phosphatase inhibitors).

That means, one or more kinases located on membranes participate(s) on phosphorylation event. Another question arises from the RAFT concept.

CagA, once injected into the cell, appears to interact with the inner leaflet of the host plasma membrane via a charge association that either directly or indirectly anchors it to the negatively charged anionic lipids in the cytoplasmic membrane. In addition, janus kinases were recruited to rafts upon H. pylori infection (Bronte, 2010, Tegtmeyer, 2010). It is presented a dynamic model of STAT3 activation, which requires the interaction of lipid raft associated proteins, H. pylori CagA and recruited JAKs with non-lipid raft receptor components to support STAT3 signaling. This study is significant since it provides insight into the possible mechanisms by which H. pylori induces gastric cancer and furthermore, it facilitates the development of novel therapeutic targets directed against bacterial induced carcinogenesis.

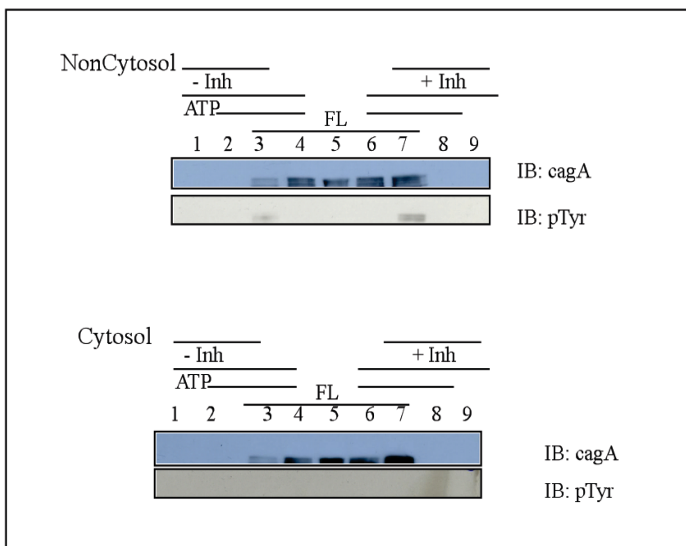


**Fig. 5** Phosphorylation with phosphorylation reaction buffer (ATP, Mg+2, phosphatase inhibitors (Asahi et al., J.Exp.Med.,2000, p.593). Positive phosphorylation in presence of non-cyt, membranes fraction, and phosphorylation reaction buffer

That lead us to conclude that phosphorylation is cellular mediated by membranes enzimes, kinases that needs substrate ATP and metal ions, Mg + 2.

Our results as literature new findings stimulate our research on study the localization of cagA at lipid-raft level, as well the investigation of phosphorylation of cagA directly using in vitro assay with specific enzymes like Src kinases, Yes and c-Src.

We could establish that cagA is located shortly after infection in lipid rafts, after performing infection of MDCK cells with Helicobacter P1 strains. Fig. 7 shows location of cagA after infection different times on lipid rafts. Figure 8 shows the cagA, their phosphorylation and the overlay correspondingly to the phosphorylated cagA .

Lipid rafts are regions of membranes with a distinct, characteristic structural composition and that appear to act as platforms to colocalize proteins involved in intracellular signaling pathways. The organization of membranes into such microdomains recognizes that, far from being randomly arranged, lipids may actually be highly organized within different parts of the membrane, and that this organization influences the way that membrane proteins are distributed (Cadler, 2007).

Lipid raft preparation was made by centrifugation in sucrose gradient (Fig. 6).

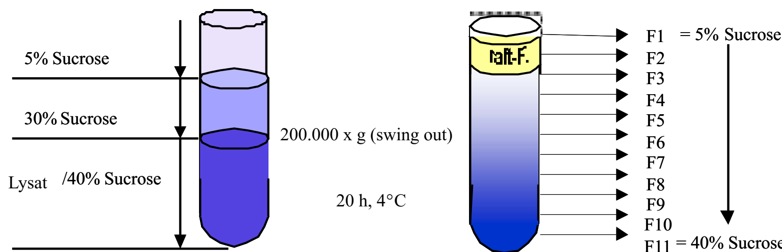


**Fig. 6** Lipid Raft preparation by sucrose gradient centrifugation.


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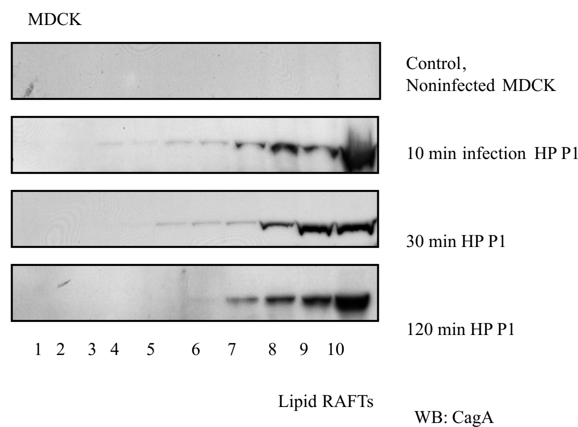


**Fig. 7** CagA in Lipid Rafts. MDCK cells were infected with P1 strain of HP at different times (10 min, 30 min, 120min), supposed to lysis and analized by SDS-PAGE and Western Blott against cagA. CAgA is loicated only on fractions 8-10 from ultracentrifugation corresponding to Rafts.

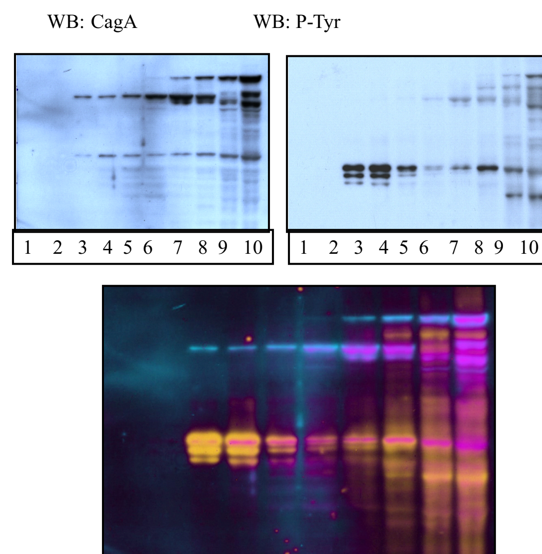


**Fig. 8** CagA in lipid rafts of MDCK cells after infection 120 minute infection time.
CagA and the phosphorylatd form can be observed.
In overlay the phosphorylated form of cagA in raft.

The results represent the localization of cagA in rafts of infected MDCK cells with HP. Simultaneously appear the nonphosphorylated and phosphorylated form of cagA in rafts.

Put all togheter, the results signify the accumulation of cagA in rafts and also the phosphorylation of this virulence factor.

## CONCLUSIONS

1. Phosphorylation of cagA in vitro is a kinase dependent process.
2. We investigate which fraction cytosolic or membranous is implied in phosporylation of virulence factor cagA of Helicobacter Pylori.
3. Phosphatase inhibitors are very important for mantaining the kinases in vitro in an active state. Whithout phosphatases inhibitors the process of phosphorylation does not take place.
4. The cytosol is not implied in phosphorylation of recombinant cagA in vitro.
5. Membranous fraction is implied in process of phosphorylation; the cytosol and membranes could be well separated using our experimental conditions.
6. Recombinant FLcagA can not be phosphorylated only by cytosol or Non-cytosol from MDCK cells. The phosphorylation take place with phosphorylation reaction buffer (ATP, Mg+2, phosphatase inhibitors). That is an evidence for supporting hypothesis that specific kinases located on cell membranes are needed to perform phosphorylation.
7. CagA is accumulated in rafts in both phosphorylated and nonphosphorylated form.


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