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STABILITY OF EFFECTOR PROTEIN cagA: A PREREQUISITE FOR HP PHYSIOLOGY AND PATHOLOGY

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ABSTRACT: Helicobacter pylori colonizes the human stomach and can induce gastritis, peptic ulceration, gastric MALT lymphoma and gastric adenocarcinoma, the second most common cause of cancer mortality in the world. We suppose that the role of effector protein CagA in pathology of HP infection is partially influenced by a very diverse and extreme environment in the stomach, with consequences on the structure and functionality of CagA and hence on physiopatology of HP.

The purpose of this study is to evaluate structural and functional CagA, the single known effector protein of Helicobacter pylori under physiological conditions.Our result indicate CagA as a very susceptible molecule to degradation dependent on medium factors. Recombinant CagA shown a very high instability and degradation under different physical-chemical factors, selective degradation especially at the functional significantly C-terminus, high sensitivity and degradation by mechanical stress and high temperature, higher temperature favouring faster degradation as lower temperature.

KEYWORDS: stability, effector protein, environment, degradation, stomach

ABBREVIATIONS: HP – Helicobacter pylori, MDCK - Madin-Darbi canine kidney cells, T3SS – type three secretion system, T4SS- type four sescretion system, SDS-PAGE sodium deodecylsulphate electrophoresys, cagA FL-cagA fullenght

INTRODUCTION

The literature data refered to the structure and function of cagA are unfortunately dissipated and limited to the mention of molecular mass, interaction with specific proteins implied in signal transduction pathway (Hitsatsune, 2008) induced by infection, the mention of biological activity like motiliy (Gauthier, 2007), cell adhesion, capacity to be translocated and subsequent phosphorylated (Tegtmeyer, 2010), dependent from the nature of H.p. strain and infected host cells.

The molecular mechanisms by wich bacterial infection leads to malignant transformation is not known at present. It has been suggested that H. Pylori is not directly mutagenic but rather acts more indirectly favouring the formation of mutagenic substances (Yamasaki, 2006).

It is supposed that the role of cagA in pathology of HP infection is partially influenced by a very diverse and extreme natural medium factors from stomach, with consequences on the structure and functionality of cagA and hence on physiopatology of HP infection.

A detailed analysis of function and structure of natural CagA is difficult to be preformed because of the variability of their natural isoforms and missing of an appropriate assay for activity determination.

Clinical evidence demonstrate that the eradication of HP increases the rate of gastro-oesophageal reflux and oesophageal cancer, revealing the protecting role of Hp as component of micro flora of the human digestive tract.

It was performed a detailed analysis of the structure and function of CagA and their physical-chemical properties whereby the recombinant CagA Full length and different deletions mutants are investigated.

The purpose of this study is to evaluate structural and functional CagA, the single known effector protein of Helicobacter pylori under natural physical-chemical conditions.

Manipulating the environment by generating a series of chaotropic agents, ionic strength, chemical destabilizators, inducing mechanical stress or physical stress by high temperature we tested in vitro proprieties of CagA like stability.

MATERIALS AND METHODS

1. Expression of recombinant CagA

Recombinant CagA, full-length and different deletions mutants, 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), GST-CagA 1-858, GST-cagA 858-1158, and intermediary fragment GST-CagA 530-858 were obtained in a pure form as illustrated in figure 1. To purifiy and eliminate the GST-tag the „Glutathione Sepharose High Performance" Amersham Biosciences (www.amershambiosciences.com) was used as descriebed by producer.

2. Native PAGE

Native PAGE (8% acrylamide) were performed. Procedure is very similarly with SDS-PAGE with difference that all used solutions do not contains SDS. Samples from rCagA FL (GST-CagA $^{1-1158}$), CagA $^{1-858}$, GST-CagA $^{1-529}$ and CagA $^{530-858}$ were analysed.

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

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3. Modifying the equilibrium polymer-momomer of rCagA

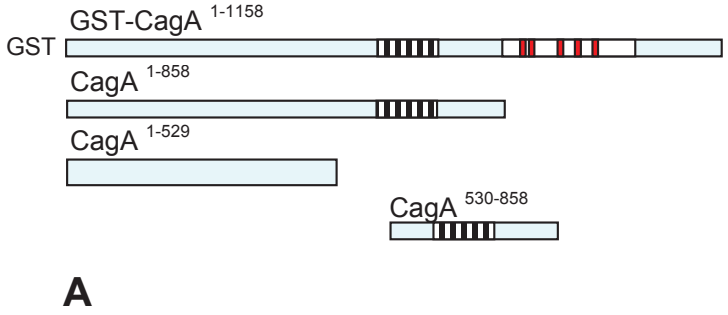
In order to disrupt dimer or polymer interactions and to achieve the pure monomer structure of rCagA, it was generated a series of chemical destabilizators of polymers, including low, nondenaturing concentration of a wide variety of detergents and chaotropic agents salt concentration: NaCl (concentration 0.1-0.5 M), detergents (N-octyl-ß-D-glucopyranosid concentration 0.05-0.25%), alkylating agents (Iodacetamide), reducing agents (Dithyothreithol). but also denaturing agents physical factors (temperature). The change in the distribution of monomers versus polymers status corresponding to different fragments of rCagA was tested by native PAGE.

4. Studies of stability of rCagA depends on temperature.

Degradation kinetic of GST cagA were folowed on SDS-PAGE on a week period. The stability was analysed at different temperatures: -20 °C, + 4°C and + 37 °C. The samples of recombinant CagA contain protease inhibitors.

RESULTS AND DISCUSSIONS

Recombinant CagA deletions mutants: rCagA fl (GST-CagA $^{1-1158}$), CagA $^{1-858}$, GST-CagA $^{1-529}$ and CagA $^{530-858}$ domains were expressed in E. Coli BL21 protease deficient strain and purifyied as GST fusion protein, as described by Materials and Methods.



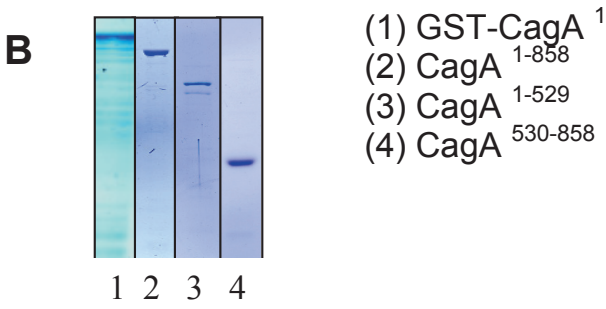


Figure 1. A schematic view of rCagA deletions mutants (A). Recombinant CagA deletions mutants: rCagA fl (GST-CagA $^{1-1158}$), CagA $^{1-858}$, GST-CagA $^{1-529}$ and CagA $^{530-858}$ domains were expressed in E. Coli BL21 protease deficient strain and purifyied as GST fusion protein, as described by Materials and Methods. The r CagA $^{1-529}$, CagA $^{858-1158}$, CagA $^{1-858}$ were isolated as cleavage product and their purity assessed by SDS-PAGE and subsequent Coomassie staining (B).

The results indicate CagA as a very susceptible molecule to degradation dependent on medium factors but capable of self protection through the binding to host chaperone in bacteria as well as after translocation in infected host cells and self interaction to form dimers.

OLIGOMERIZATION

Analysis under native condition shows a high degree of oligomerization

The purified proteins corresponding to construct CagA $^{1-529}$, CagA $^{530-858}$, CagA $^{1-858}$ (fig. 1) were first examined by chromatography on a **gel filtration** column. Because of the limitation of gel filtration to estimate the molecular mass of macromolecules (mobility of protein in gel filtration column is affected by molecular weight and shape) was difficult to distinguish between the momomere, dimere and polymere forms. The unusual mobility of the molecular forms of CagA constructs reflects probably the fast equilibrium dimers-polymers or momomers-dimers in solution. A distinct separation and characterization of rCagA molecular forms in solution using gel filtration could not be done partially because of the limitation of resolution of gel filtration and also because of the very high instability and cleavage of rCagA during the chromatographic separation step as show the Dotblot analysis of chromatographically separated fractions.

The coexistence of monomers, dimers and polymers forms of CagA in solution were certified using other native method. The expressed CagA fragments corresponding to the CagA $^{530-858}$ and CagA $^{1-858}$ was checked for their properties under **native PAGE** (8% acrylamide) (fig. 2).

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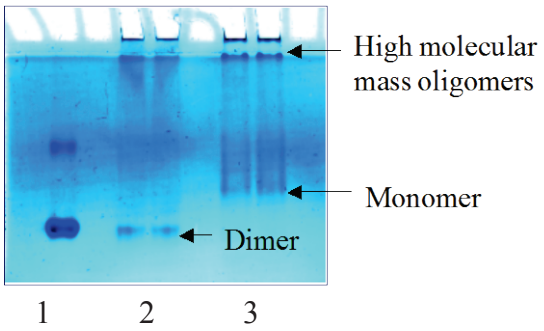

Figure 2. Distribution of rCagA as monomer, dimer and oligomer. RCagA was separated shortly after preparation under native PAGE (8% polyacrylamide) followed by Coomassie staining. Lane 1- BSA as molecular mass control; lane 2- CagA $^{530-858}$; lane 3- CagA $^{1-858}$

Construct CagA $^{530-858}$ exhibit a mixture of dimers, tetramers and high molecular mass oligomers in solution. Construct CagA $^{1-858}$ shows a equilibrium of monomers and high molecular mass oligomers in solution.

The used protein concentration was 30 µM for r CagA $^{1-858}$ and 20 µM for r CagA $^{530-858}$, that means that the observed high molecular weight oligomers cannot be attributed to the unspecific aggregation because of a high protein concentration.

Modifying the equilibrium polymer-momomer of rCagA

CagA $^{530-858}$ domain appears as dimer and high molecular mass oligomers when experiments were conducted at different temperature (4-50°C), salt concentration (NaCl concentration 0.1-0.5 M), detergents (N-octyl-ß- D-gluco pyranosid concentration 0.05-0.25%), alkylating agents (Iodacetamide), reducing agents (Dithyothreithol).

Under similar treatment Construct CagA $^{1-858}$ exhibit predominantly high molecular weight bands localized at the top of gel and minor amount of monomer. The change in the distribution of monomers versus polymers status corresponding to different fragments of rCagA was tested by native PAGE. The equilibrium monomere-polymere is strongly moved in the favour of the polymers form for all investigated fragments of rCagA under tested condition. Taken together these results indicate the missing of an effective physical-chemical method for the stabilization of rCagA monomers or destabilization of oligomers. The aggregation of recombinant proteins were also reported, as negative property espessialy in farmaceutical formulation (Arakawa, 2006).

Ionic strength, pH, detergents, reducing and alkylating agents

The stability of rCagA is sensitive to pH changes. RCagA is more stabile after incubation at pH 7,5 as at pH 11 and 4°C, while rCagA38 is more stable at pH 9,5 as at pH 6,5. Raising the temperature at 37°C, the intact rCagA disappear totally already after 2 days of incubation (data not shown). The samples was analysed under Native PAGE.

The effect of detergents, as destabilizer of dimer structure was tested. N-octyl-ß-D-glucopyranosid increasing concentration (00,5-0,25%) had no effect on distribution of monomer/dimer equilibrium. Analog when the sample was treated with increasing amount of NaCl (0.1-1.0 M) or with alkylating agent iodoacetamide or DTT. Summarizing all these observation, no chemical method (pH, salts, common stabilizators, denaturing agents) in vitro against degradation and stabilization of monomers was founded.

Stability

Stability of rCagA was checked in order stabilize the monomer structure of rCagA and to find a way for destabilization of oligomers of rCagA. In addition we have estimated the effect of extreme milieu of stomach on the structure of rCagA.

Stability of rCagA depends on temperature.

Higher temperature favours a faster degradation as lower temperature (fig.3). The degradation kinetic of GST-FL-rCagA show a very rapid degradation even for 24h, with the totally cleavage and disappearance of CagA after 7 days.

The cleavage products were analysed using LC-MS after performing in gel digestion. The only stable fragment of fusion protein is the GST tag that remain intact. Degradation occurs selectively at CagA level and specially at C-terminus domain. It was tested if the splitting of CagA is the consequence of presence of some protease from E.Coli. The selective identification of rCagA fragments show no protease between the degraded fragments. Moreover the experiment was conducted in presence of protease inhibitors, so that the proteolitic action of proteases is excluded.

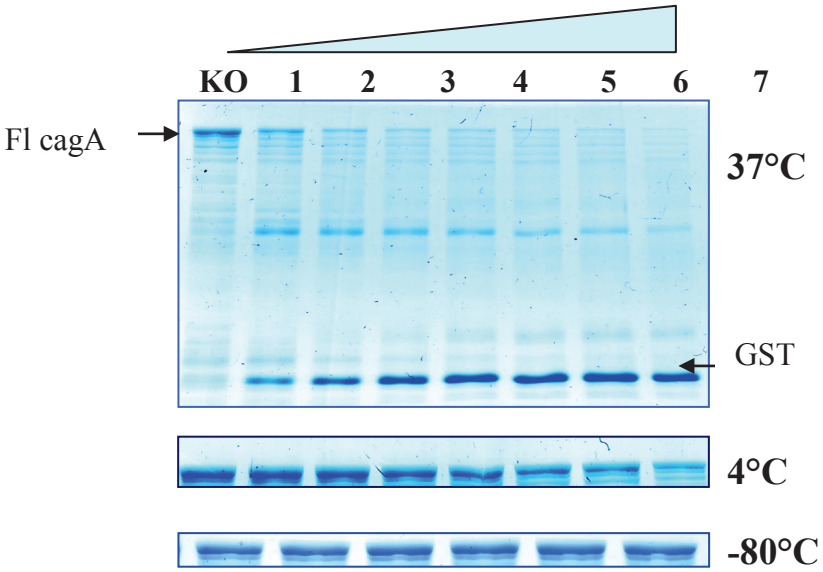

Figure 3. Time course degradation of rCagA dependent on temperature
RCagA was treated under different physical-chemical factors various time. The samples were separated by SDS-PAGE and subsequently Coomassie staining. As control is designed the sample at the start point. FL-rCagA, as GST fusion protein, was incubated at 37°C or 4°C for 7 days. Totally degradation of FL-rCagA can be noted after 7 days at 37°C compared with 4°C. GST tag (arrow) remains intact.

No tripsin similary activity of rCagA identified

Because the fragments of rCagA resulted after the temperature dependent degradation are selectively splitted at Lys and Arg, trypsin sensitive cleavage points, we checked the trypsin like activity of rCagA. We incubated the recombinant FL-CagA with a peptid containing a selective cleavage site for trypsin at 37°C for 1h. The sample was analysed by MALDI-MS. No fragmentation effect on peptid was observed.

The time course degradation of GST rCagA deletions mutants with environment (puffers)

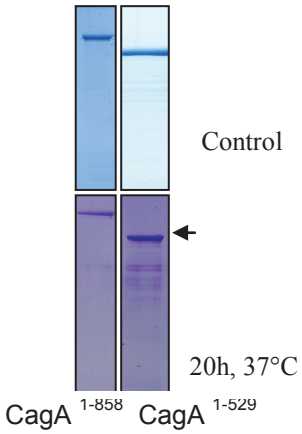

Fig. 5. The time course degradation of GST rCagA deletions mutants, CagA $^{530-858}$, CagA $^{1-858}$ subjected to incubation in 100 mM Hepes buffer + 100 mM NaCl at 37°C for 20h (bottom pictures). The arrow represent the position of non-degraded construct. The degradations fragments of rCagA was checked by in gel digestion and MS analysis. The fragmentation occur preponderantly at C-terminal region of rCagA.

CagA $^{530-858}$ domain appears as dimer and high molecular mass oligomers when experiments were conducted at different temperature (4-50°C), salt concentration (NaCl concentration 0.1-0.5 M), detergents (N-octyl-ß-D-glucopyranosid concentration 0.05-0.25%), alkylating agents (Iodacetamide), reducing agents (Dithyothreithol).

Under similar treatment Construct CagA $^{1-858}$ exhibit predominantly high molecular weight bands localized at the top of gel and minor amount of monomer. The change in the distribution of monomers versus polymers status corresponding to different fragments of rCagA was tested by native PAGE. The equilibrium monomere-polymere is strongly moved in the favour of the polymers form for all investigated fragments of rCagA under tested condition. Taken together these results indicate the missing of an effective physical-chemical method for the stabilization of rCagA monomers or destabilization of oligomers.

Action of visible light

We tested if the stability of rCagA is affected by visible light, due to the literature observation that the HP are killed by exposure to harmless levels of visible light.

rCagA FL was incubated at room temperature and exposed to visible light, variable time (3, 6, 24 h). The samples were subjected to SDS-PAGE followed by Coomassie blue staining. No photosensitivity of CagA could be observed during the experiment (Fig 6).

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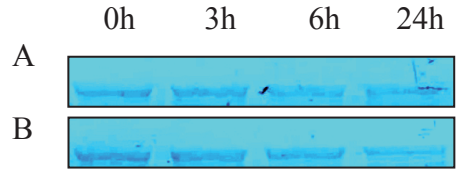

Fig. 6. Effect of visible light on degradation of FL-rCagA. FL-rCagA was exposed to visible light (A) or kept at dark (B) at room temperature for 24h. The quantitative densitometry scanning show no effect of visible light on rCagA degradation.

Extraction of CagA from natural medium or their expression as recombinant protein leads to degradation, probably because of the missing of the natural medium factors like proteins (corresponding bindingsproteins) or the appropriate milieu (pH, temperature, electrolytes, etc). Recombinant CagA interact in vitro with itself leading to the formation of dimers, tetramers or highly molecular mass oligomers, or with others proteins implied in their stabilization.

Excepting the influence of translocation of CagA trough T4SS (Bourzac), the cycle phosphorylation/ dephosphorylation seems to be implied in the stability of CagA in vitro, promoting probably in vivo the conversion of CagA shortly after translocation in a physiological active and stable conformation.

One hypothesis about the stability of cagA is that the conformation of rCagA expressed in E. Coli is not the native conformation and that specifically chaperone could promote the natural folding or in opposite the missfolding of newly synthesized proteins either in E. Coli as well in HP or HP infected cells.

Covacci has reported since 1993 different cagA molecules with different molecular masses. It is not known if the multiple cagA identified originate from a single protein cagA through posttranslational modifications or are the rezult of expression more genes.

Apart from the stability of cagA with a key role of in HP infection, this study reach a fundamental problem of farmaceuticals formulation (Cromwell, 2007), where the stability therapeutical proteins is required.

CONCLUSIONS

The detailed analysis of physico-chemical properties of rCagA reveals the following characteristics of this recombinant protein in solution.

A. High degree of oligomerization

1. high degree of oligomerization as shown the analysis under native condition
2. Oligomeric rCagA is very stabile, no physico-chemical method for stabilization of monomers found.

B. High degree of instability under different chemical conditions

1. selective degradation at CagA level, specially at C-terminus domain.
2. no auto-proteolitic or tripsin similary activity found.
3. Instability of rCagA dependent on temperature, higher temperature favours a faster degradation as low temperature
4. No chemical method against degradation founded (pH, salts, common stabilizators)

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