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LOCALIZATION OF CagA EFFECTOR PROTEIN OF HELICOBACTER PYLORI IN INFECTED EPITHELIAL CELLS

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ABSTRACT

Epidemiological studies have shown the role of CagA-positive Helicobacter pylori (Hp) in development of atrophic gastritis, peptic ulcer disease and gastric carcinoma. CagA effector protein of bacterial pathogen Helicobacter pylori is translocated in host cell via TFSS and subsequently tyrosine phosphorylated. Both phosphorylated and non phosphorylated CagA activate multiple signal transduction pathways promoting disruption of cell-cell contacts, migration and the typical hummingbird phenotype (Bouzrac, Highasi). This study proposed to establish the location of CagA in epithelial cell in culture after infection with Hp. The interaction of extracellular protein CagA from Hp with membrane and cytoskeletal proteins of host cell is to expected, taking in account the multiple contribution of cagA in signal transduction and cell shape modifications, that implied membrane respectively cytoskeletal proteins.
The experimental results give one image regarding the traffic of CagA in host cells. According to these data we conclude that Cag A is first, predominantly associated with membranes and cytoskeleton.
Key words: infection, cell culture, effector, membranes
Abbreviations: Hp – Helicobacter pylori; MDCK - Madin-Darby canine kidney cells; TFSS-type four secretion system, rCagA-recombinant CagA, IB-immunoblotting, PBS-phosphat buffer saline, SDS- PAGE- sodium dodecyl suphate poliacrylamide electrophoresys, S-PEK - ProteoExtract® Subcellular Proteome Extraction Kit

INTRODUCTION

Helicobacter pylori is a highly successful bacterial pathogen stomach typical that affects more than half of the population. Infection is correlated with digestive pathology like chronic gastritis, peptic ulcer (75% of cases) duodenal ulcer (90% of cases) (Ernst, 2000) and sometimes with 2 cancer forms: MALT (mucosa associated lymphoid tissues) and adenocarcinoma (Blaser, 1998). Recent studies have revealed that cancer related diseases account for more than 700000 deaths each year (Hatekayama), being the second reason of death cancer related. A direct correlation was signalized between incidence of Helicobacter pylori infection and frequency of gastric cancer (Crew, 2006). The relationship between Helicobacter pylori infection and pancreatic cancer has been investigated with contradictory results (Lindkvist). *Helicobacter pylori* were also identified in dental plaque, although a correlation between presence in dental plaque and stomach could not be done (Chitsazi).

CagA is the most studied bacterial virulence factor of Helicobacter pylori. CagA protein is codified by a 40-kpb region of ADN named "cag pathogenity island" (PAI). This gene encode proteins, components of type four secretion system (TFSS), regarded as a molecular syringe that inject bacterial effectors molecules into the cytoplasm of host cells (Selbach). Presence of cagA is correlated with the most aggressive cancer types. Recent studies have revealed the presence of cagA protein in about 70% Helicobacter pylori strains, but the frequency depends on geographical area. Gastric cancer is the most common cancer in several areas of the world, most notably Japan, Korea, and China. In Japan the incidence of gastric carcinoma is almost ten times higher than in USA. In most areas, the men are almost twice as high as in women (Hatekayama, 2009).

CagA is translocated into gastric epithelial cells and localizes to the inner surface of the plasma membrane, in which it undergoes tyrosine phosphorylation at the Glu-Pro-Ile-Tyr-Ala (EPIYA) motif by Srk kinases family. Membrane localization of CagA may be required for cagA phosphorylation (Highasi, 2005). Recruitment of CagA in membrane is supposed to be essential for activity as pathogen of Helicobacter pylori. Membrane association of CagA requires the EPIYA –containing region but is independent of EPIYA tyrosine phosphorylation (Highasi, 2005).

Translocation and subsequent phosphorylation of cagA is reported as the main inducer of morphological modifications with the observed dramatic elongation, referred as "hummingbird phenotype" because of the beak-like extensions produced in the elongated cells (Bourzac). Although there is a correlation between expression of cagA and Helicobacter pylori virulence, their function remain not elucidated. The wide variety of cagA effects, a different molecular forms phosphorylated/ non-phosphorylated, existence of numerous fragments with not identified function arise a lot of questions refers to role of this effector protein in host cells immediately after infection.

This study proposes to investigate the location and intracellular trafficking of CagA in infected MDCK cells with Helicobacter pylori P1. To determine the location of CagA in host epithelial cells short time after infection is one of the points that should be elucidated in order to establish the pathological mechanisms of infection.

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MATERIALS AND METHODS

Chemicals

We have purchased ProteoExtract® Subcellular Proteome Extraction Kit from Calbiochem (Cat. No. 539790) and the other chemicals from Santa Cruz Biotechnology: polyclonal antibody against CagA after immunization, monoclonal antibody anti c-Jun, anti ERK, anti E-cadherin, anti-beta-Tubulin and anti-caveolin.

METHODS

Infection with Hp P1

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. One day before infection, cells were washed with medium without serum, 5 ml twice, and then incubate over night in medium without serum. Infection with Helicobacter pylori strain P1 was realized at bacteria density of $5x10^8$ in PBS. Infection time was 4 hours at 37 °C.

Selective extraction of protein subcellular fractions with ProteoExtract® Subcellular Proteome Extraction Kit (S-PEK)

The protocol is published by www.calbiochem.com and consists in the following steps (figure 1). S-PEK enables the differential extraction of proteins according to their subcellular localization. The special mild S-PEK procedure yields proteins in their native state. S-PEK takes advantage of the differential solubility of certain subcellular compartments in special reagent mixtures preserving the structural integrity of the subcellular structures before and during the extraction. With Extraction Buffer I cytosolic proteins are released (fraction 1, F1). Subsequently, membranes and membrane organelles are solubilized with Extraction Buffer II, without impairing the integrity of nucleus and cytoskeleton (fraction 2, F2). Next, nucleic proteins are enriched with Extraction Buffer III (fraction 3, F3). Components of the cytoskeleton are finally solubilized with Extraction Buffer IV (fraction 4, F4).

We have obtained cellular fraction correspondingly to cytosolic proteins (F1), membrane protein extract from membranes (F2), nuclear proteins (F3) and the proteins of matrix (F4). The extraction was monitorized by contrast phase microscopy.

Testing the efficiency of extraction of proteic subcellular fractions using organelle specifically marchers

Proteins extracted corresponding to fractions F1-F4 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.

Test of cagA localization in subcellular fractions

Samples of protein fractions F1-F4 extracted from infected P1 MDCK cells were supposed to SDS-PAGE and immunoblotting against cagA. The pattern of CagA distribution was followed in infected cells compared with adequate controls (F1-F4): MDCK cells not infected. The presence of cagA was tested by immunoblotting. The control was CagA protein recombinant.

Extraction with hypoosmotic buffer

MDCK cells after infection with Helicobacter pylori P1 strain different times (1h,2h,150 minutes) were extracted with hipo osmotic buffer. As control were used not infected cells. 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 µl 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. The supernatant represents the cell lysat.

RESULTS AND DISCUSSIONS

Action mechanisms of Helicobacter are not entire understood. It is known that transduction of signals in epithelia host cells is influenced. The process begin with "injection" of cagA from Helicobacter pylori through secretion system type four, a multiprotein complexes with enzymatic function and also chaperone characteristic important presumably in stabilization of CagA efector protein. CagA is injected in host cells, specifically phosphorylated at characteristic domains. Phosphorylation affects the metabolic pathways intracellular, with implications on the shape (cell elongation) by direct interaction with cytoskeleton components, changes in functions of intercellular junctions and gene transcription.

Phosphorylation of cagA is the key event in initiation of pathogen response. That's why it was extensively investigated. In 2002 it was establish that Src an enzyme from Src kinases family is involved in phosphorylation of CagA.

Investigation of cagA localization in proteic fractions after extraction with ProteoExtract® Subcellular Proteome Extraction Kit (Figura 4) reveals the predominance of effector protein in membrane/organelle fraction (F2) and cytoskeleton (F4). There are data in literature that summarize information about interaction of cagA with other receptor for tyrosin kinases (RTF), e.g. c-Met that acts as an effector of phospholipase C (PLCν). The reaction products of PLCν as PIP2 (phosphatidyl inositol di-phosphate) activate

gelsolin, profilin and cofilin with subsequently changes on actin dynamic (Chuirin, 2003). Identification of CagA in cytosolic fraction (figure 4) is to expect taking in account actin modifications.

Phosphorylation of cagA sems to be regulated through inactivation of own kinase C-Src by feedback regulation. Inactivation occurs after direct linkage of phosphorylated cagA, final product of C-terminal kinase (Csk) that can phosphorylates at tyrosine residues and inactivate in such way member of Src family kinases (Tsunami, 2003)..

In such way is inactivated phosphorylation of cagA and other proteins of host cells such ezrin and cortactin, that can initiate elongation in AGS cells. Both ezrin and cortactin mediate actin organization during the contact of pathogens as E.coli and Salmonella. The cell morphology changes, so called "hummingbird phenotype" is associated with cell scattering and increased cell motility (Highasi, 2005).the molecule SHP-2 can also induce the hummingbird phenotype but the morphological modification requires membrane association of SHP-2, and simultaneously of cagA , taking in account that the both protein interact each others. This observation was made by Highasi (2005) and indicates that the plasma membrane recruitment is essential for SHP-2 function and suggested that the primary role of cagA in host cells is to translocate cellular protein that SHP-2 from the cytoplasm to the membrane and activate them. It is suggested that cagA mimic mammalian scaffolding adaptor protein, such Gab family members.

A schematic representation of tissue cell culture MDCK with S-PEK is given in the figure 2. MDCK cells are represented after successive extractions with correspondingly extractions buffers on contrast phase microscopy.

Figure 3 shows MDCK cells after extraction of cytosolic proteins (a), after extraction of membrane/ organelle proteions (b), or after the extraction of nuclear proteins (c). The purity of proteins fraction was checked by SDS-PAGE and immunoblotting. Incubation was made with specifically antibody for each cellular/ subcellular fraction as described by materials and methods. The protein patterns of the respective fractions are clearly distinct. (F1-4: Fraction 1-4). The efficiency of extraction was tested using specifically marchers (figure 3).

Presence of marcher proteins characteristic for each subcellular fraction demonstrate that separation was made in accordance with subcellular localization. Earlier publication reported that association of cagA with membranes is important in pathogenic response of bacteria in gastric epithelial cells. Our result refers to MDCK epithelial cell line model of infection with Helicobacter pylori. For infection we chose the P1 strain, a cagA positive strain.

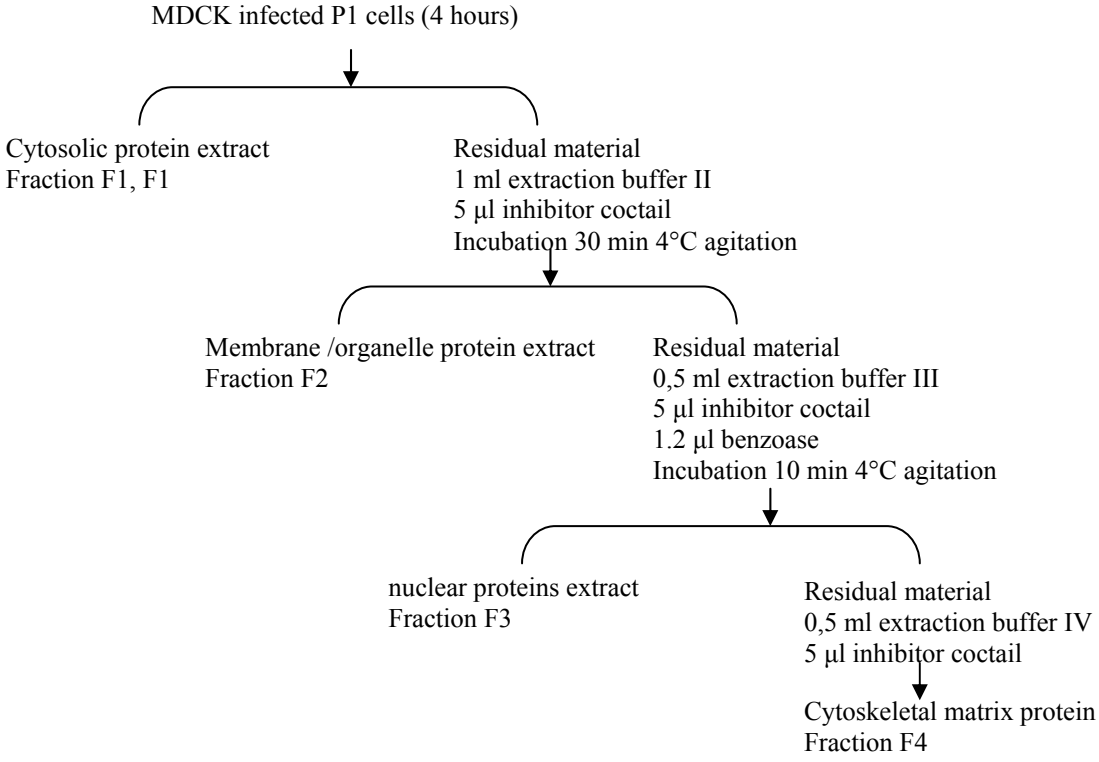

Figure 1. Schematic representation of S-PEK extraction of adherent tissue culture cells

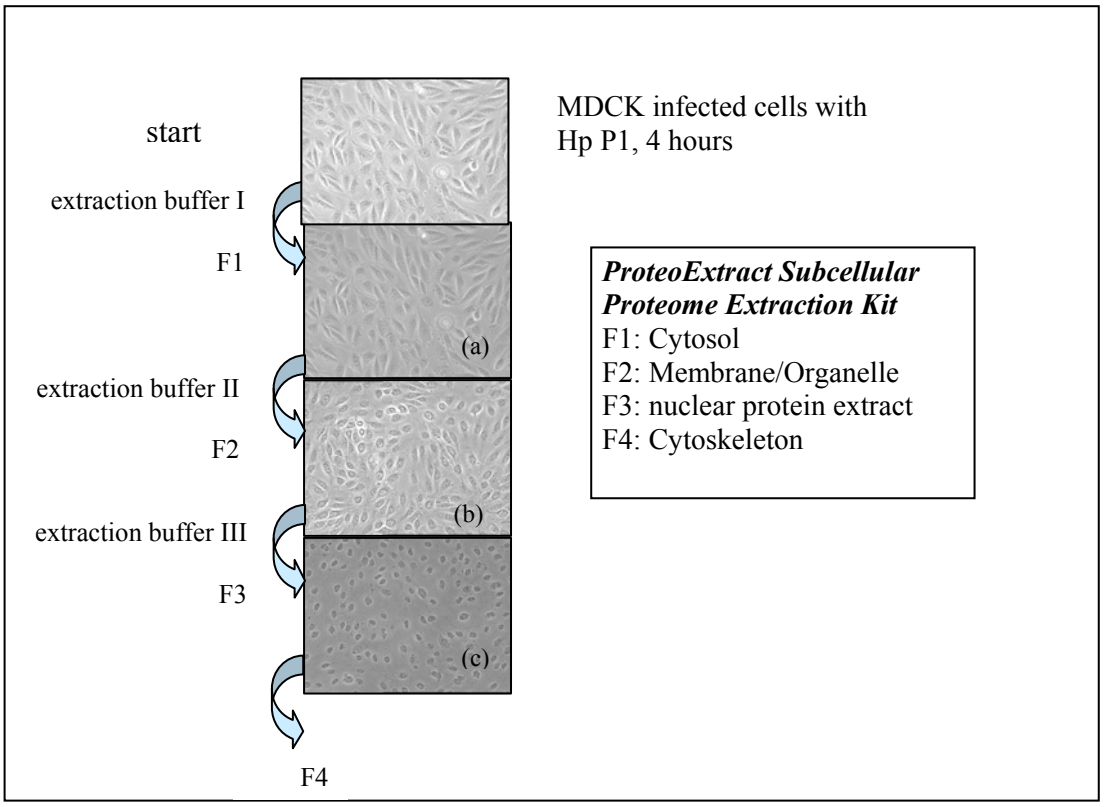

Figura 2. Schematic representation of S-PEK extraction of adherent tissue culture cells. MDCK cells after successive extractions with correspondingly extractions buffers on contrast phase microscopy

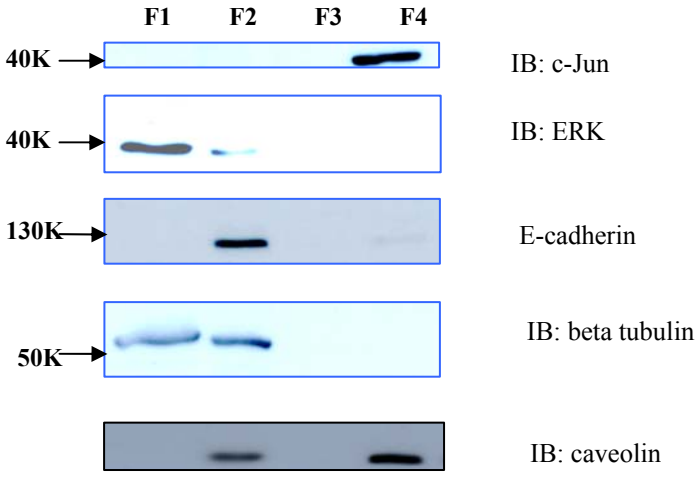

Figura 3. SDS-PAGE of subcellular fractions after S-PEK extraction of adherent tissue culture cells demonstrating that protein patterns of the respective fractions are clearly distinct. (F1-4: Fraction 1-4). The efficiency of extraction was tested using specifically marchers. With arrows are represented molecular masses.

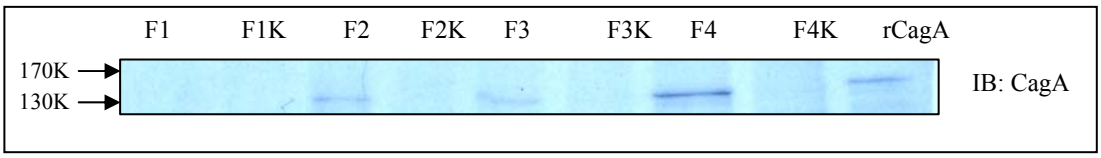

Figure 4. Localization of CagA. F1 fraction F1 (cytosolic), F1K (control), F2 membrane/ organelle fraction, F2K control, F3 nuclear extract, F3K control, F4 cytoskeleton proteins, F4K control, recombinant CagA

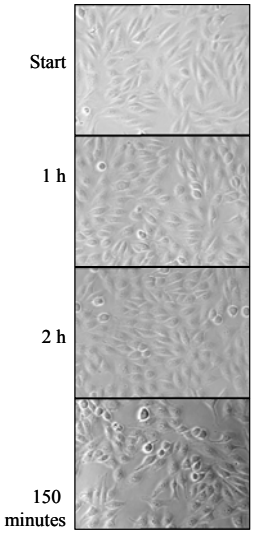

Figure 5. MDCK cells after infection different times with Helicobacter pylori P1 strain and extraction with hypo osmotic buffer

Another way to obtain subcellular fractions is the lysys in hypo osmotically buffer. MDCK cells infected was supposed to extraction and the both fractions cytosolic (Cyt) and non cytosolic (nonCyt) were tested for the accuracy ofextraction usingthe characteristicmarchers disscused in figure 3. Results are shown in figure 6 in comparission with the extractractions with S-PEK.

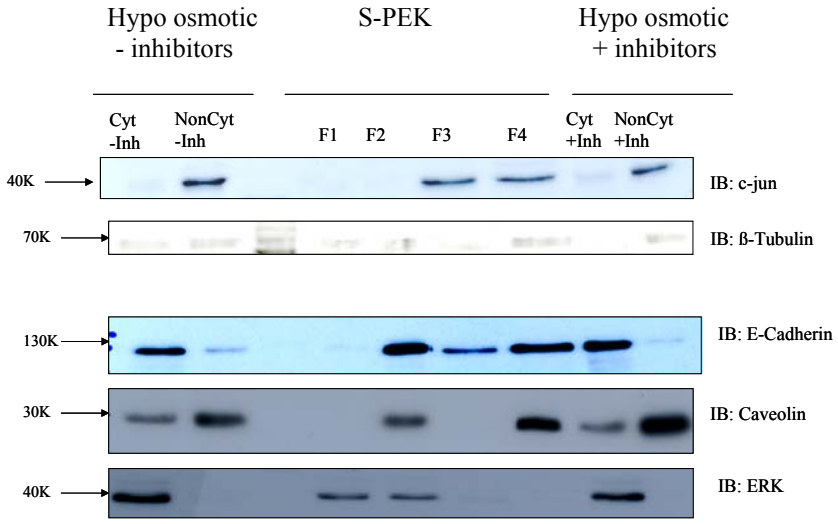

Figure 6. Distribution of marchers for subcellular localization. Comparison between extraction with S-PEK and hypo osmotic buffer. Legend: Cyt- cytosol fraction; noncyt-nont cytosolic fraction; Inh-protease inhibitors. F1-F4 correspond to extraction with S-PEK.

CONCLUSIONS

The localization of cagA effector protein of Helicobacter pylori was under investigation. The cellular components (membrane/ organelle, cytosolic, nuclear, extracellular matrix, cytoskeleton) were obtained using the commercially available kit ProteoExtract Subcellular Proteome Extraction Kit from Calbiochem. Infected MDCK cell in culture were investigated after infection 4 hours with Helicobacter pylori P1. The results suggested the co localization of cagA in the highest proportion on cytoskeleton (fraction F4) and membranes (fraction F2). Effector protein was also identified in little amount in nuclear fraction (F3). It is to expected the interaction of an extracellular protein (from pathogen agent) first, shortly after infection, with cell membranes and cytoskeleton, taking in account the multiple role of CagA proved in numerous publications, especially on signal transduction, in which are membranes implied, in modification of cell shape, with mediating effect of cytoskeleton. One image of cagA trafficking in host cells short after infection can be made. The conclusion of experiment is that cagA is located preponderantly after 4 hour infection associated with membranes and cytoskeleton in host MDCK infected cells. This result is in accordance with experimental results from literature regarded cagA effects.

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