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ORIGINAL ARTICLE

Expression of *CagL* from *Helicobacter pylori* and Preliminary Study of its Biological Function

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Abstract *Helicobacter pylori* (*H. pylori*) is a highly successful human-specific gastric pathogen, infecting over half the world's population. Virulent *H. pylori* isolates harbour the cytotoxin-associated genes pathogenicity island (cag-PAI), the majority of which have no known function. In this study, we used cell infection assay and reverse transcriptase PCR, identified that CagL recombinant protein, one of the cag-PAI proteins, induced GES-1 cells to express cytokine IL-8. Then we performed western blot and translocation assay. Our result showed CagL polyclonal antibody counteracted translocation of CagA. This will provide a foundation for the further studies on its biological function.

Keywords *Helicobacter pylori* · T4SS · CagA · CagL

Introduction

Helicobacter pylori (*H. pylori*) is a gram-negative, spiral-shaped, microaerophilic bacterium that colonizes the human gastric mucosa and is recognized as the causative agent of chronic active gastritis, gastric and duodenal ulcers or lymphoma of the MALT (mucosa-associated lymphoid tissue) system, and is a risk factor for the development of adenocarcinoma [1–3]. This microorganism has been categorized as class I carcinogen by the World Health Organization [1]. The pathogenesis of *H. pylori* mainly depends on the exposure of several bacterial factors, including cytotoxin-associated gene A (CagA), the type IV secretion system (T4SS), vacuolating cytotoxin A, outer inflammatory protein A and several adherence factors, to the host [4–6]. Due to their pivotal role in *H. pylori* pathogenesis, these factors are currently being intensively studied to elucidate how they induce specific host responses.

Cag-PAI, a 40 kb stretch of DNA, encodes components of a T4SS. This T4SS forms a pilus for the injection of virulence factors into host target cells such as the CagA oncoprotein [7, 8]. This is accomplished by a specialized adhesin of the pilus surface, the CagL, which binds to and activates integrin $\alpha_5\beta_1$ receptor on gastric epithelial cells through an arginine–glycine–aspartate motif. This interaction triggers CagA delivery into target cells as well as activation of focal adhesion kinase and Src [9]. Furthermore CagL is a potential antigen candidate for *H. pylori* vaccine.

In this study, the *cagL* from *H. pylori* has been cloned and expressed, moreover, we have found that the CagL recombinant protein could induce GES-1 cells to express cytokine IL-8 mRNA and participate in translocation of cagA protein. The results will provide a foundation for the further studies on *H. pylori* pathogenesis.

Materials and Methods

Bacterial Strains and Growth Conditions

Helicobacter pylori strain NCTC 11637 was cultured on agar plates containing 5 % fetal bovine serum (FBS)

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(BioMerieux) and incubated for 48 h at 37 °C under a microaerophilic atmosphere. Bacteria were harvested in PBS, pH 7.4 *H. pylori* strains were grown in Brucella broth with 5 % FBS for 24 h, with shaking under a microaerophilic atmosphere. Then they were harvested by centrifugation.

Preparation of DNA Template

Genomic DNA of *H. pylori* was prepared by the routine phenol–chloroform method and DNase-free Rnase treatment. The obtained DNA was dissolved in TE buffer, and its concentration and purity were determined by ultraviolet spectrophotometry.

Polymerase Chain Reaction

Primers were designed to amplify the sequence of *cagL* from strain *H. pylori* NCTC 11637 based on the published sequences of strain *H. pylori* 26695. The sequences of *cagL* sense primer with an endonuclease site of *Bam*HI and antisense primer with an endonuclease site of *Xho*I were 5′-GAT GGATCC GAA GATA TA AC AA G C G GTTT-3′ and 5′-GCCCTCGAGTTTAACAATGATCTTACTTGA-3′ respectively. The parameters for PCR were 94 °C for 5 min, ×1; 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s, ×30; and then 72 °C for 10 min, ×1. The results of PCR were observed under UV light after electrophoresis in 10 g/l agarose, prestained with ethidium bromide.

Cloning and Sequencing

The amplified target DNA fragment from *cagL* was cloned into pGEM-T vector (pGEM-T -cagL) by using the T-A Cloning kit according to the manufacturer's instruction. A professional company (BBST) was responsible for nucleotide sequence analysis of the inserted fragment. The *cagL* target fragment and PET28a(+) were recovered for ligation. The recombinant expression vector PET28a(+)-*cagL* was transformed into *E. coli* BL21DE3, which was named as PET28a(+)-*cagL*-BL21 (DE3). The target *cagL* fragment inserted in PET28a(+) plasmid was sequenced again.

Expression and Identification of the Fusion Protein

PET28a (+)-*cagL*-BL21 was cultured in LB medium at 37 °C and induced by IPTG at concentrations of 1.0 mmol/l. The bacterial were harvested at different time of 2, 3, 4, 5 h. The supernatant and precipitate were separated by centrifugation after the bacterial pallet was ultrasonically broken (300 V, 5 s × 3). The molecular mass and output of CagL were measured by SDS-PAGE. The expressed CagL was collected by Ni–NTA affinity chromatography. The mouse anti-His and HRP-labeling sheep anti-mouse IgG were used as the first and second antibodies, respectively, to identify the CagL fusion protein by Western blot.

Preparation and Identification of the Polyclonal Antibody

Rabbit antiserum was prepared against the CagL purified protein in a 6 weeks old New Zealand White rabbit. The rabbit was immunized with 1 ml of purified protein (0.5 mg/ml) emulsified in 1 ml of Freund's complete adjuvant and followed immunization with 1 ml of purified protein (0.5 mg/ml) emulsified in 1 ml of Freund's incomplete adjuvant at an interval of 14 days. After four times of injection, 1 ml of the protein solution was then given intravenously at weekly intervals for a further 4 weeks. The rabbit was bled 1 week after the last immunisation and the serum was collected. ELISA assay was used to detect the titer of the antibody. Antibody was also identified by Western blot.

Cell culture and Infection Experiment

GES-1 were grown in DMEM medium containing 20 % heat-inactivated FBS in a humidified 5 % CO_2 atmosphere. Cells were seeded in tissue culture plates before infection. Eighty percent confluent monolayers were washed in PBS and were incubated in serum and antibiotic-free medium. Before infection, *H. pylori* was neutralized by CagL polyclonal antibody serum at different concentrations of 5, 10, 20 %V/V) and incubated for 4 h at 37 °C under a microaerophilic atmosphere. The bacteria were added to the host cells at a multiplicity of infection of 300 for 6 h. Cultures were maintained at 37 °C under a 5 % CO_2 atmosphere. As a control, cells were infected with PBS and non-neutralization *H. pylori* for the indicated time periods. All experiments were repeated 3–4 times.

Preparation of Cell Lysates and Western Blotting

The infected cells were harvested in lysis buffer(20 mmol/l Tris-hydrochloride (pH, 7.5), 150 mmol/l sodium chloride, 1 % Triton X-100, 1 % NP-40, 3 mmol/l sodium vanadate, 20 mmol/l sodium fluoride, 1 mmol/l phenylmethylsulphonyl fluoride, 10 mg/ml aprotinin, and 10 mg/ml leupeptin) and separated by SDS-PAGE, transferred onto PVDF membranes. To analyze translocation of CagA, a monoclonal CagA antibody (Aalto Bio Reagents Ltd., Dublin, Ireland) and Horseradish peroxidase-conjugated anti-mouse (Amersham, Germany) antibody were used as the first and second antibodies by Western blot and detected with the kit system for ECL (Amersham, Germany). As a control, the anti-β-actin antibody was used.

RNA Isolation and Reverse Transcriptase PCR

After stimulation, total RNA was isolated using the RNA-easy Midi Kit (Qiagen, Germany) as recommended by the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed into single-stranded cDNA with Superscript II RT (Invitrogen, Germany) and oligo(dT)primers. Amplification of IL-8 (forward: 5′-TACTCCAAACCTT TCCAC CC-3′, reverse: 5′-CCTACAACAGACCCACACA AT-3′) and GAPDH (forward: 5′-CCACCCATGGCAAATCCATG GC-3′, reverse: 5′-TCTAG ACGGCAGCGGCAGGTCAG GTCCACC-3′) was described before. PCR products were visualized by ethidium bromide staining after agarose gel electrophoresis.

Statistical Analysis

Data were analyzed using Student's *t* test and were expressed as mean values of at least 3 independent experiments ± standard deviations. Differences were considered to be statistically significant at $P < 0.05$.

Results

Construction of Recombinant Plasmid and Restriction Enzyme Confirmation

The fragment of *cag*L was cloned into pGEM-T vector and expression vector PET28a(+), resulting in recombinant plasmids named pGEM-T-*cagL* and PET28a(+)-*cagL*. The recombinant plasmids were all digested by *Bam*HI and *Xho*I simultaneously. Then digestive products were visualized on 1 % agarose gel electrophoresis.

The pGEM-T-*cagL* and PET28a(+)-*cagL* plasmids were identified by PCR, 2 restriction enzymes digestion (*Bam*HI and *Xho*I), and single restriction enzyme digestion with *Bam*HI, respectively. The results showed that an approximately 650 bp band was amplified by PCR, band approximately 650 bp and 3000, 650 and 5,400 bp were observed following 2 restriction enzymes digestion for pGEM-T-*cagL* and PET28a(+)-*cagL* plasmids, respectively, and about 4000, 6000 bp bands were observed by single restriction enzyme digestion for pGEM-T-*cagL* and PET28a(+)-*cagL* plasmids, respectively. The sequencing results of both cloning plasmids showed that the band was specific, which was the same as the designed results.

Sequence Analysis

The homologies of nucleotide and putative amino acid sequences of the cloned *cagL* gene compared with the published *cagL* sequences were from 96 to 98 % and from 97 to 99 % respectively. The potential antigenic epitopes of the CagL was analyzed by DNASTAR7.0 software. The results showed that this protein has a plurality of structural domain of antigenic activity.

Expression and Identification of Target Fusion Proteins

Gel automatic scan analysis showed that the final concentration of IPTG was 1 mmol/l and the expression of CagL rose remarkably after 4 h of induction, which accounted for 48 % of total bacterial proteins. The result of Western blot showed the clearly identifiable band, which was similar to that predicted (Fig. 1).

Polyclonal Antibody Specifically Recognizes CagL Protein

Six immunizations with the CagL were given to rabbit. Antisera containing CagL antibody was harvested from rabbits 59 days after the primary immunization day. ELISA results showed that the titer of the antisera was 1:320000. Western blot results showed that these antisera were able to recognize recombinant CagL (Fig. 1).

Polyclonal Antibody Counteracts Translocation of CagA

We evaluated whether there was any difference in the levels of CagA translocation into *H. pylori*-infected GES-1 cells before and after neutralization of polyclonal antibody. Whole-cell lysates were prepared and analyzed by Western blotting to quantify the amount of CagA protein delivered into cells. When *H. pylori* were pretreated with different concentrations of polyclonal antibody, the level of CagA translocation decreased in a dose-dependent manner (Fig. 2). Compared

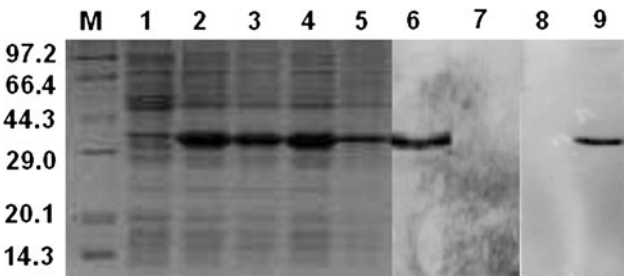

Fig. 1 The effect of inducing time on the expression amount of recombinant CagL and analysis of specificity of polyclonal antibody against CagL Expression of CagL was induced by 1 mmol/l IPTG. *M* protein mark, *1* negative control (PET28a(+) vector), *2–5 E. coli* BL21(DE3) containing PET28a(+)/*cagL* recombinant plasmid induced with IPTG after 2–5 h respectively, *6* Western blot analysis of CagL recombinant protein, *7* negative control, *8* Western blot analysis of serum of negative control, *9* Western blot analysis of polyclonal antibody against CagL

with nontreated group, there was a significantly smaller amount of CagA translocation. Together, these results suggest that the function activity of CagL is required to mediate the translocation of CagA by the T4SS efficiently.

CagL Induces Expression of IL-8

It was previously reported that *H. pylori* leads to IL-8 induction via the NF-kB signaling pathway in cells. Furthermore, the CagL protein is a specialized adhesin of the pilus surface, which binds to and activates integrin $\alpha_5\beta_1$ receptor on gastric epithelial cells. Thus, we sought to assess whether CagL affects IL-8 expression, we analyzed IL-8 mRNA in non-stimulated and CagL stimulated GES-1 cells. A significantly higher level of IL-8 mRNA was detected in cells stimulated with CagL in a dose-dependent manner (Fig. 3), indicating that CagL can mediate immune response of cells, which may contribute to translocation of CagA.

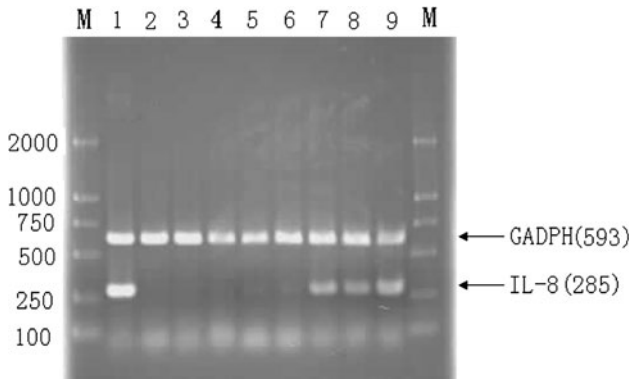

Fig. 3 IL-8 mRNA expression in GES-1 cells stimulated with PBS, LPS and recombinant CagL protein. GES-1 and LPS, PBS, different concentrations of recombinant CagL were cultured for 4 h, respectively. *M* DNA Marker DL2000, *1* Positive control (GES-1 cells co-cultured with LPS), *2* GES-1 cells co-cultured with PBS, *3* GES-1 cells co-cultured with the extractive of bacterium transformed with PET28a(+), *4–9* GES-1 cells co-cultured with different concentrations of recombinant CagL (1, 5, 10, 15, 20 and 30 μg/ml)

Discussion

T4SS are highly versatile secretion machineries that transfer effectors from bacteria to either bacteria of the same or different species, or from bacteria to fungi, plants or mammalian target cells. The assembly of T4SS transporters in the inner and outer membranes of the bacteria [10–15] and the transport of substrates through the assembled T4SS is well documented [16]. However, almost nothing is known about how T4SS substrates are delivered across the cell membrane in their eukaryotic host. It is also unclear whether components of the T4SS specifically recognize their target cell before injecting effector molecules. Indeed, Most T4SS of different pathogens do not translocate their effectors into the culture supernatant [4, 12]. This suggests that functional activation of these T4SS requires a signal from the host cell, for example, the interaction with a specific receptor. Host cell integrins were recently shown to directly interact with the *H. pylori* CagL and are so far the only T4SS receptor known. Integrins are cell adhesion receptors that mediate cell–cell, cell–extra cellular matrix and cell–pathogen interactions by binding to Arg-Gly-Asp (RGD) motifs [9]. Integrin function is dynamically regulated by processes termed outside-in and inside-out signalling [17]. Similarly, CagL, a protein highly conserved among pathogenic *H. pylori* strains, is the only cagPAI-encoded gene product that contains an RGD motif which mediates binding of the pilus to integrin $\alpha_5\beta_1$ and is required for injection of CagA [9]. Binding of CagL to integrins induces local membrane ruffling, indicative of a general effect on membrane dynamics which is the first T4SS effect on host cells. After established the T4SS injection needle, *H. pylori* also affects the actin cytoskeleton, transcriptional responses and cell-to-cell junctions. Therefore, whether the CagL-mediated signalling might facilitate type-IV secretion by altering membrane dynamics is an intriguing proposition to be investigated further.

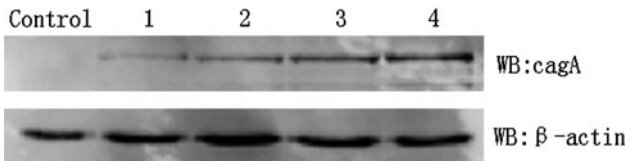

Fig. 2 The result of CagA's translocation after neutralization GES-1 cells were infected with *H. pylori* strain and *H. pylori* neutralized by CagL polyclonal antibody for 6 h, respectively. Then, the translocation of CagA was compared between them. *Control* GES-1 cells without *H. pylori* treated, *1–3* GES-1 cells co-cultured with *H. pylori* of CagL polyclonal antibody neutralization (CagL polyclonal antibody density degrade gradually), *4* GES-1 cells co-cultured with *H. pylori* without neutralization

The CagL-integrin-mediated CagA injection is crucial for deregulation of focal adhesions. However, rearrangement of the actin cytoskeleton leading to cell migration appears to be mainly independent of CagA, but requires a functional T4SS [18–21]. In particular, *H. pylori* mutants that do not express the CagL are unable to stimulate cell migration, while *H. pylori* strains that are deficient for CagA still activate motility to a certain extent [18]. This led to the hypothesis that CagL-mediated stimulation of the β1 integrin/FAK/Src pathway is involved in the cytoskeletal rearrangement. The interaction between CagL and integrin may lead to dissociation of the E-cadherin/p120^{ctn}/β-catenin complex from the actin cytoskeleton occurred rapidly by interrupting its binding to α-catenin, which may aberrantly activate p120 and regulates expression of the carcinogenic effector, mmp-7.

In this study, we have cloned and sequenced *cagL* from *H. pylori*. The polyclonal antibody against *cagL* was obtained. We have demonstrated that CagL recombinant protein can induce host cells to express cytokine IL-8 mRNA and CagL polyclonal antibody can inhibit translocation of CagA protein, the date indicate that CagL can mediate immune response of cells and the function activity of CagL is required to mediate the translocation of CagA by the T4SS efficiently. Therefore, CagL might be a novel drug target for combating the severe gastric diseases caused by cagPAI-positive *H. pylori* strains. In a word, our study provided a foundation for the further studies on cagL biological function and *H. pylori* pathogenesis.

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