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C-terminal domain of CagX is responsible for its interaction with CagT protein of *Helicobacter pylori* type IV secretion system

Gopal Jee Gopal [a,b,*], Jagannath Pal [a,c], Awanish Kumar [d], Gauranga Mukhopadhyay [a,*]

[a] Special Centre for Molecular Medicine, Jawaharlal Nehru University, New Delhi, India
[b] Department of Biochemistry, M.S. University of Baroda, Vadodara, Gujarat, India
[c] Department of Medical Oncology, Dana Farber Cancer Institute, Boston, MA, USA
[d] Department of Biotechnology, National Institute of Technology, Raipur, Chhattisgarh, India

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ABSTRACT

Helicobacter pylori are the well known human pathogen associated with gastric cancer and peptic ulcer. Pathogenesis is mainly due to the presence of 40 kb *cag*PAI (*cag* Pathogenicity Island) region that encodes the type IV secretion system (TFSS) consisting of a cytoplasmic part, a middle part/core complex (spans from inner membrane to outer membrane), and an outer membrane associated part. CagX and CagT are two important proteins of TFSS that have homology with virB9 and virB7 of *Agrobacterium tumefaciens* TFSS. In this study, we have shown that the CagX and CagT interact directly by using co-immunoprecipitation of endogenous CagX and CagT and MBP pull down assay. We further authenticate this observation using yeast two-hybrid assay and co-expression of both the protein coding gene in *Escherichia coli*. We also observed that the C-terminal region of CagX is important for CagT interaction. We reconfirm that CagT depends on CagX for its stabilization. These observations could contribute in overall visualization of assembly and architecture of TFSS because protein–protein interactions among Cag proteins are likely to have an important role in assembly. Thorough understanding about architecture and mechanism of action of cag-TFSS may lead to design controlled drug delivery system.

1. Introduction

Helicobacter pylori are the major cause of chronic gastritis and play an important role in the pathogenesis of peptic ulcer, gastric adenocarcinoma and gastric lymphoma [1–3]. *H. pylori* is a Gram-negative, spiral shaped, flagellated slow growing bacteria which lives for decades in the extreme acidic environment of human stomach [4–5]. For colonization and survival in the human stomach, it expresses the multisubunit enzyme urease [6–7]. Patients with gastritis, ulcers and malignancies are often infected with *H. pylori* type I strains, which are characterized by the presence of a 40-kb DNA segment called the cag pathogenicity island (*cag*-PAI). The *cag*-PAI of *H. pylori* contain around 30 open reading frames (ORF) and *cag*A gene (cytotoxin-associated antigen A) is a marker for this pathogenicity island, which is absent in *H. pylori* type II strains [8]. Some of the *cag*-PAI genes share significant homology with the virulence (*vir*) genes of the *virB*/*virD* complex of type IV secretion systems of *Agrobacterium tumefaciens*, *Bordetella pertussis*, *Legionella pneumophila*, and *Ehrlichia chaffeensis* and encodes a functional type IV secretion system that injects the CagA effector molecule into cytosol of gastric epithelial cells [6,9–10]. Type IV secretion system (TFSS) are ancestrally related to the bacterial conjugation system and are versatile transporters of proteins and/or nucleic acids (effector molecules) across the bacterial membrane to the extra cellular space or into eukaryotic target cells [11–13].

In the present study some of the key questions related to *H. pylori* TFSS have been addressed especially, the interaction between CagX and CagT. It has been already shown that in the absence of cagX or cagT gene of respective isogenic null mutant strain, there was neither translocation of CagA protein into the host cell nor induction of IL8 occurred [14]. Busler et al. showed, through yeast two hybrid assay that CagX interact with CagY [5]. Recently, it has also been observed that CagX and CagT form pilus of *H. pylori* [11]. To show the interaction between these two proteins, we have used different molecular biology and biochemical tools i.e. MBP pull down assay, co-immuno precipitation, yeast

* Corresponding authors at: Department of Biochemistry, M.S. University of Baroda, Vadodara, Gujarat, India (G.J. Gopal).
E-mail addresses: gopaljigopal@rediffmail.com (G.J. Gopal), garunga@hotmail.com (G. Mukhopadhyay).

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two hybrid and co-expression assays and found the interaction between these two proteins. We have also observed that C-terminal region of CagX is important for its interaction with CagT.

2. Materials and methods

2.1. H. pylori culture

H. pylori strain 26695 was grown on brain heart infusion (Difco) agar supplemented with 7% fetal calf serum, 0.4% campylobacter growth supplement and DENT (Oxoid). Plates were incubated at 37 °C for 48 h in a micro-aerophilic atmosphere (GasPack-100, BBL).

2.2. Isolation of genomic DNA

Genomic DNA was isolated from *H. pylori* as described by Simor et al. [15] with minor modification. Briefly, *H. pylori* cells were scraped from BHI-agar plate's and suspended in PBS. Cells were centrifuged and pellet was washed first with PBS then with TE. The washed pellet was re-suspended in 160 μl/10 mg weight cells of TE (50 Mm-Tris–HCl, 5 mM EDTA) and lysozyme (500 μg/ml) for 30 min at room temperature. 1% SDS and 0.05 mg/ml of RNase-A was added and incubated again for 60 min. at 37 °C. Proteinase K was added (final concentration 0.5 mg/ml) and incubated over-night at room temperature. Equal volume of phenol:chloroform:isoamyl (25:24:1) was added, and centrifuged at 12 K for 10 min. The aqueous layer was separated and DNA was precipitated by adding 0.1 volume of sodium acetate and 2.1 volume of ethanol. Precipitate was centrifuged followed by washing with 70% ethanol. Precipitated DNA was dissolved in TE.

2.3. Cloning of genes and their deletion derivatives

To generate a clone of cagT devoid of signal sequence, the ORF of hp0532 without signal sequence was amplified using the primer pair FN532Δ22Nw/RX532w from Hp26695 genomic DNA, digested with NcoI and XhoI, and cloned between NcoI and XhoI sites of pET 28a to make pET-cagT plasmid. Protein produced has polyhistidine tag at its C-terminal termed Ns-CagT-His(CagT). *cagX* gene and its deletion derivatives were cloned in pMAL-c2X vector to get CagX protein conjugated with N-terminal MBP tag (maltose binding protein). Name of constructs and primer pair used for amplification of gene for cloning are mentioned in Supplementary Table. For construction of middle deletion mutant of cagX (hp0528ΔM105; M105 = middle 105 residues were deleted) in pMAL-c2X (pMAL-528ΔM105) the sequences upstream and downstream of deleted portion (+91 to +660) and (+776 to +1569) were amplified separately using primer pairs F528ΔN30B/R528ΔC302P (F & R = forward and reverse; B & P = BamHI and PstI) and F528ΔN325P/R528H (P & H = PstI and HindIII) respectively and ligated with digested vector together (Supplementary material). Primer pairs and restriction sites used for cloning of other genes and their derivatives are mentioned in Supplementary Tables.

2.4. Construction of mutator plasmid for creation of null mutant strain

For creation of cagX, cagA and cagT null mutant strain, pJP90, pWS30 and pJP95, plasmid respectively were used for transformation of *H. pylori* 26695 strain (kind gift from Prof. Haas, Germany). For creation of *cagM* null mutant strain, mutator plasmid pBS-cag7ΔcagM/CatGC was constructed following published scheme and protocol [14]. Briefly, *cag7* sequence was PCR amplified from *H. pylori 26695* genomic DNA using cag7leftN/cag7rightK (N & K = NotI and KpnI respectively) primer pair by Pfu polymerase and cloned into pBluescript between KpnI and NotI sites resulting in pBScag7. Next, plasmid pBSCag7 was copied excluding sequence encoding *cagM* (*hp0537*) by inverse PCR strategy using 537FB/537RX (B & X = BamHI and XhoI) primer pair (Suppl. Table 1). The inversely amplified PCR product was digested with BamHI and XhoI and ligated with the terminator less CatGC cassette excised from pKS-CAT plasmid. *E. coli DH5α* competent cells, transformed with the ligated product were platted on ampicillin containing LB-agar plate. Positive clones were first selected on chloramphenicol plate and finally by double digestion of plasmids isolated from chloramphenicol resistant colonies. Transformed *H. pylori* strains were further tested for deletion of *cagM* by testing expression of the gene by Western blotting using anti-CagM antibody. Wild type *H. pylori 26695* were used as a positive control.

2.5. Recombinant CagT and CagX protein expression

E. coli BL21 (DE3) strain containing plasmid pET-cagT was grown in LB medium containing 50 μg/ml Kanamycin till OD_{600} 0.6. Thereafter, 1 mM IPTG was added to induce the expression of recombinant CagT and incubated further for 3 h at 37 °C. *E. coli* BL21 or DH5α strain containing plasmid constructs of cagX gene and its deletion derivative cloned in pMAL vector were grown in LB medium containing 100 μg/ml of ampicillin till OD_{600} 0.6. Thereafter, 1 mM IPTG was added to induce the expression of recombinant CagT and incubated further for 3 h at 37 °C.

2.6. Co-expression of CagX and CagT in E. coli

For co-expression of recombinant proteins CagX and CagT, the published protocol [18] with minor modifications was followed. pACYC-duet plasmid vector is compatible with pET-28a. Competent BL21 (DE3) cells were transformed with pACYC-*cagX*/pET-cagT. Transformed *E. coli* cells were selected on chloramphenicol/kanamycin (pACYC/pET) double antibiotic containing LB-agar plate. The protocol for co-expression was same as expression of single gene except culture media contained double antibiotics instead of single antibiotic.

2.7. Polyclonal antibody generation against CagT

Protein was extracted from the insoluble material with solubilization buffer (150 mM NaCl, 10 mM phosphate buffer pH-8.0, 1% Triton X-100, 10 mM β-ME and 1 mM PMSF). In the subsequent step, the extract was subjected to ammonium sulfate precipitation at 29% saturation followed by centrifugation at 10 K for 20 min at 4 °C. The precipitated material was resuspended in 10 mM Tris–HCl buffer pH 7.4 and separated on 10% SDS–PAGE. Protein bands were visualized by treating the gel with ice-cold 0.1 M KCl solution as described by Kosman et al. with minor modification [17]. The desired protein band was excised from the gel and pulverized by pushing the gel piece through the orifice of a 1 ml pipette tip. The pulverized gel pieces containing 250–200 μg of the protein were suspended in PBS and Freund's complete adjuvant until micelles were formed and injected sub-cutaneously into New Zealand White rabbit for primary immunization. After a second booster dose, blood was collected for anti serum. Crude serum was diluted 10 times with TBST and pre-cleared with acetone dried *E. coli* powder and used at a dilution of 1:10,000 in Western blot analyses.

2.8. Co-immunoprecipitation assay

Either recombinant proteins or *H. pylori* extracts were used for co-immunoprecipitation assays. 40 μl packed volume of *Hp*26695 cells was re-suspended in 400 μl of lysis buffer (20 mM Sodium