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

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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

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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

phosphate buffer pH 7.4, 150 mM NaCl, 2 mM EDTA, 10% glycerol, and 6 μl of 100×-protease inhibitor cocktail) containing 2% DDM (*n*-Dodecyl β-D-maltoside), lyses by sonication and centrifuged at 10 K for 20 min. Supernatant was collected and diluted with equal volume of IP-buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and 0.1 mM PMSF). The sample was pre-cleared by adding pre-immune rabbit serum and protein-A Sepharose. Each pre-cleared sample was divided into two equal parts; 2 μl of pre-immune rabbit serum was added to one part, and 5 μl affinity purified anti-CagT or 1.0 μl anti-CagX antibody was added to the other part and incubated for two and half hours at 4 °C on a vertical rocker at 4 °C. Following incubation, 15 μl of protein-A Sepharose was added to each sample and incubated for additional 1 h. Following brief centrifugation at 5000*g*, bound protein was released by boiling the beads with SDS-sample buffer, subjected to SDS–PAGE, followed by Western blotting using desired antibody. Further, membrane was stripped of with primary antibody and re-probed by second antibody.

2.9. Yeast two-hybrid assay

For yeast two hybrid assay, modified pGAD424/pGBT9 MATCHMAKER GAL4 yeast two-hybrid system was used [19] and named as pGAD-C1 and pGBD-C1. The first step in yeast two-hybrid screen is to construct a bait plasmid (pGBD-C1-x; where x = gene of interest) and prey plasmid (pGAD-C1-y; where y = gene of interest) that express the proteins of interest as a fusion to the DNA-BD (DNA binding domain) and DNA-AD (activation domain) domain respectively. *H. pylori* genes *cagT* (*hp0532*), *cagX* (*hp0528*), *cagF* (*hp0543*), and *cagA* (*hp0547*) were cloned in both the vectors following standard protocol for reciprocal interactions. A selective pair of positive plasmids was introduced (as described below) into the reporter yeast strain (*AH109*); positive transformants were selected on SD/–Trp/–Leu. Selected yeast colonies from each transformation were tested for restoration of His and ade auxotrophy by streaking on triple or quadruple drop out SD/leu/trp/ade/his agar plate.

2.10. MBP pull down assay

MBP-tagged CagX was expressed in *E. coli* strain DH10β, where as polyhistidine-tagged CagT was expressed in Bl21 (DE3). Cell pellets containing MBP-taged proteins were resuspended in suspension buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF and 10% glycerol). Thereafter, 0.2 mg/ml lysozyme was added to the cell suspension and cells were lysed by sonication. The lysed cell suspension was centrifuged at 10 K for 20 min at 4 °C. The resulting supernatants were collected and used for pull-down experiment. His-CagT was not soluble and recovered from inclusion bodies by sarcosine treatment. Cell lysates containing MBP-tagged CagX and MBP alone were mixed with pre-equilibrated Amylose resin and incubated for 1 h in vertical rocker at 4 °C. Resin bound proteins were washed twice with binding buffer followed by blocking with 0.1% BSA in binding buffer. In the subsequent step, the measured amount of sarcosine solubilized recombinant His-CagT was added to the respective Amylose resin bound MBP-tagged proteins/MBP, and incubated for 1 h at 4 °C. Protein-bound resins were washed 3–4 times and analyzed by SDS/PAGE followed by Western blotting. Anti-His and anti-MBP (NEB) antibodies were used for detecting His-CagX (prey) and MBP/MBP-tagged bait.

2.11. Western blotting

SDS–PAGE was carried according to Laemmli's method [16]. Western blotting was performed by transfer of protein separated in SDS–PAGE on PVDF (polyvinylidene difluoride) membrane using semi-dry transfer system (BIORAD) according to standard protocol. Membrane was blocked with 5% Bloto (Genotech Inc, USA) in TBST, incubated with 1:10,000 dilution of HRP-conjugated secondary antibody (Bio-Rad, USA) and detected with ECL.

3. Results

We have shown that CagX is the part of high molecular weight complex of TFSS using gradient Blue Native PAGE and this complex may form due to homooligomerisation [20]. But we could not rule out the possibility of heterooligomerisation. To discover the possibility of another interacting protein which could participate in complex formation, we explored the interacting partner of CagX. It has been observed that, CagX homologue (virB9) in *Agrobacterium* interacts with CagT homologue (virB7). Based on this information, we investigate whether CagX and CagT in *H. pylori* also interact.

3.1. CagX directly interacts with CagT

As shown in Fig. 1A, both anti-CagX and anti-CagT antibodies were found to co-immunoprecipitate CagT and CagX respectively from the detergent solubilize wild-type *Hp* extract. No immunoprecipitation of the mentioned proteins was, however, observed in the case where control antibody was used (rabbit pre-immune serum). As expected, in the case of CagT deletion mutant (*HpΔcagT*), anti-CagT/anti-CagX antibody failed to immunoprecipitate CagT. Together these results suggest that CagX and CagT interact with each other under physiological condition. However, it was not clear from the results whether the interaction is direct or if accessory factor(s) are involved.

In order to test whether these proteins interacts directly, recombinant MBP tagged CagX and polyhistidine tagged-CagT (C-terminal his_6; devoid of N-terminal signal sequence) were prepared and MBP-pull-down experiments were performed. We observed that MBP tagged recombinant CagX successfully pulled recombinant CagT as shown in Fig. 1B, but MBP alone could not. This result shows direct interaction of CagX and CagT. However, questions may be raised regarding use of detergent solubilize recombinant CagT in the above experiment. To circumvent this dilemma, these two proteins were co-expressed in *E. coli*. We used co-expression system because when two interacting proteins are co-expressed they might regain their native conformation and

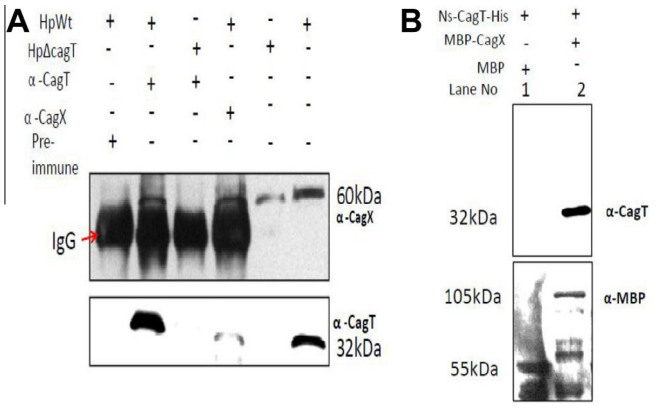

Fig. 1. Western blot showing CagX/CagT interaction (A) Co-IP of CagT/CagX from wild-type Hp and HpΔcagT mutant extracts by anti-CagX/anti-CagT antibody and (B) MBP pull down assay using MBP-CagX and His-CagT (Ns-N terminal short deletion) recombinant protein. Arrow indicates position of CagX. Pre-imm stands for control IgG. Antibodies used in Western blotting are indicated.

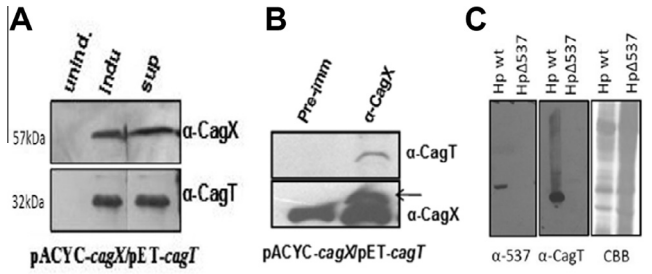

Fig. 2. Western blot showing (A) coexpression of CagX and CagT and their solubility. Plasmids used to co-express the genes are indicated. (B) Co-immunoprecipitation (Co-IP) of CagX from co-expressed total extract. Antibodies used in Western blotting are indicated. Pre-immune serum used as control antibody. (C) CagT was observed as unstable in Δ*cagM* mutant strain.

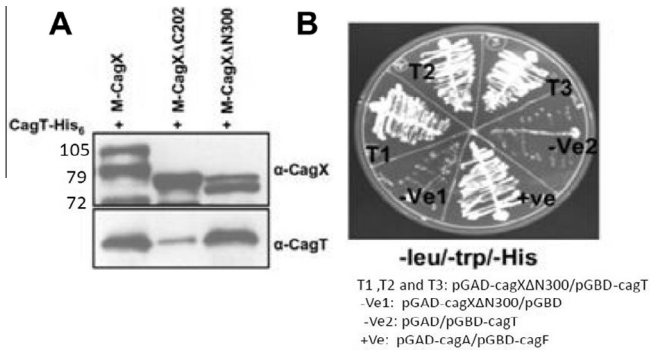

Fig. 4. CTD of CagX is required and sufficient for CagT interaction. (A) MBP pull-down analysis shows that CTD of CagX (222 amino acids) alone is sufficient for CagT interaction. Mutated CagX used are marked. Antibodies used in Western blotting are indicated. (B) Yeast two hybrid assay shows that CTD of CagX (222 amino acids) alone is sufficient for CagT interaction.

solubility and therefore remain associated in solution. As shown in Fig. 2A, both the proteins were recovered in soluble form. When immunoprecipitation experiments was performed on co-expressed proteins using anti-CagX antibody, CagT was also precipitated along with CagX suggesting again direct interaction between the proteins (Fig. 2B). During the course of the present work, a group of researcher published a report claiming that CagT interacts with CagX through an intermediate CagM (Hp0537) [21]. To test this claim the interaction was re-examined in Δ*cagM* isogenic *Hp26695* mutant strain. In Δ*cagM* mutant strain, CagT was observed to be unstable (Fig. 2C).

3.2. C-terminal domain of CagX is important for CagT interaction

The direct interaction between CagX and CagT observed above prompted us to identify the minimal region of CagX involved in protein–protein interaction. Therefore, a series of CagX deletion constructs were made having MBP tag at the N-terminal end as described in Section 2. We were able to express all deletion constructs (Fig. 3A). Next, MBP-pull-down experiments were performed using whole-cell extracts prepared from *E. coli* containing expressed recombinant CagX deletion derivatives. MBP was used as negative control as described in materials and methods. We observed that recombinant CagT was efficiently interacted with all the CagX derivatives except M-CagXΔC200, where interaction efficiency was relatively weak (Fig. 3B). This observation indicated that C-terminal domain (CTD) of CagX might be required for CagT interaction. To substantiate the result co-immunoprecipitation experiments were performed using same combination of recombinant proteins. As shown in Fig. 3C, all the CagX derivatives except M-CagXΔ200 were able to co-immunoprecipitate CagT efficiently therefore suggesting the importance of the C-terminal 200 amino acids stretch in CagT interaction.

3.3. C-terminal region of CagX alone is sufficient for interaction with CagT

MBP-pull-down experiments were performed using M-CagXΔN300 (residues 301–522) and M-CagXΔC200 as bait and recombinant CagT as prey. We observed that unlike M-CagXΔC200, M-CagXΔN300 pulled CagT efficiently (Fig. 4A). Taken together, these results strongly suggest that C-terminal region of CagX is required and sufficient for CagT interaction. As shown in Fig. 4B, only pGAD-*cagA*/pGBD-*cagF* (positive control) and pGAD-*cagXD*/pGBD-*cagTD* (test; 3 colonies) showed growth but not negative controls (coexpression of pGAD-*cagXD*/pGBD (−ve 1) and pGAD-/pGBD-*cagTD* (−ve 2) yet again suggesting the importance of C-terminal last 220 amino acid stretch of CagX in CagT interaction.

3.4. CagX stabilizes and helps in translocation of CagT

As mentioned above CagT and CagX are homologues of virB7 and virB9 of *Ag* type IV secretion system. In *Ag* type IV system, virB9 is stabilized by virB7 following heterodimer formation by disulfide linkage [22–23]. In order to test the stability of CagX and CagT in isogenic mutants of *Hp26695,* their expression level was tested in defined knockout strains. We have observed less expression of cagT in absence of cagX (Supplementary Fig. 1).

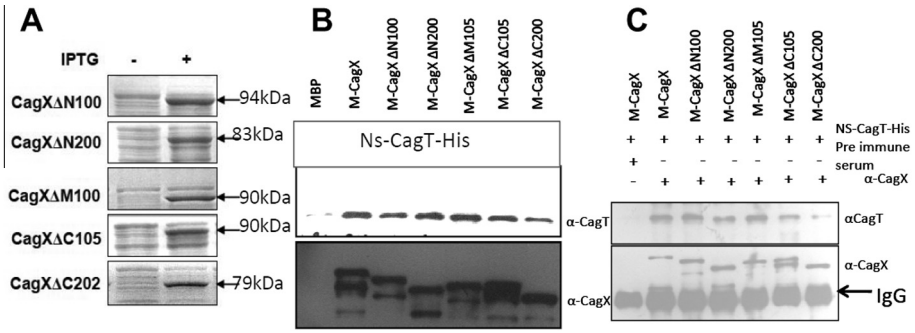

Fig. 3. (A) Expression profile of indicated recombinant CagX deletion derivatives. Extents of deletions are marked on the right side of CagX. Gel was stained with coomassie blue. (B) MBP pull-down analysis showing interaction between recombinant CagT and indicated CagX deletion derivatives. MBP was used as a negative control. Antibody used in Western blotting is indicated. (C) Co-IP showing interaction between recombinant CagT and indicated CagX deletion derivatives.

4. Discussion

Previously we have shown that CagX is a part of high molecular weight core complex due to homooligomerisation [20]. Here we have checked the existence of its hetero-oligomerisation (interaction with other protein) since that high molecular weight is only due to self association is less likely to occur. In *A. tumefaciens* CagX homolog virB9 interact with virb7 (a CagT homolog). As mentioned earlier that CagX is a predicted virB9 homologue of *Agrobacterium* and share considerable sequence homology at the C-terminal region with the *Agrobacterium* counterpart. In *Agrobacterium,* virB9 interacts with virB7 through disulfide linkage and forms heterodimeric complex [22–23]. However, sequence analysis of CagX (devoid of signal sequences) and CagT (virB7 homologue) reveals no cysteine residue. It was reported earlier that although cysteine residues of virB9 and virB7 are required for heterodimer formation, the disulfide linkage is not necessary for their functions as with tumor formation [23]. To test the interaction between CagX and CagT; CagX and/or CagT were immunoprecipitated from DDM solubilized cell extracts prepared from wild-type *Hp* 26695/*HpΔcagT* isogenic mutant strains using either anti-CagX or anti-CagT antibody (Fig. 1A).

In this study some of the key questions related to *H. pylori* TFSS have been addressed especially interaction between CagX and CagT. We have also determined that C-terminal region of CagX is important for interaction. As we know that CagX and CagT homolog remain associated in *Agrobacterium* TFSS. Therefore, we have investigated whether CagX and CagT also interact in *H. pylori* or not. We observed CagX pulled CagT from total *H. pylori* extract and *vice versa*, but we could not ruled out the possibility of bridging protein, through this experiment. To rule out the possibility of indirect interaction, we used MBP pulled down experiment using recombinant protein. Here we again observed that CagX successfully pulled the CagT protein. We could not use MBP tagged CagT for reciprocal pull down because MBP tagged CagT was unstable. This experiment clearly shown that CagX directly interact with CagT. To authenticate our observation, we have used coexpression assay followed by immunoprecipitation. We observed that CagX and CagT both are in soluble fraction when co-expressed while goes in inclusion bodies when expressed alone. This observation indicated that CagX–CagT has some physical association which help in solubility of protein by masking the exposed (when expressed alone) hydrophobic patches. Again we successfully pulled CagT (Fig. 2B) from this co-expressed total extract using anti-CagX serum and showed direct interaction between CagX and CagT.

Kutter et al. claimed that CagX and CagT don't interact directly but CagM playing a bridging role between them [21]. This observation is in contrast to our observation of direct interaction. We wanted to carry out co-immunoprecipitation of CagX-CagT from CagM null mutant strain but we could not get expression of CagT in absence of CagM. The absence of CagT in CagM null strain was not unusual because Fischer et al. also have had same observation [14]. We have extended our research to find out domain responsible for interaction, when it was established that CagX directly interact with CagT by multiple ways. *In-vitro* MBP pull-down experiment and Yeast two-hybrid assay indicated that C-terminal 200 residue is important for interaction. Reciprocal study could also be carried out to explore the interacting domain of CagT but MBP tagged CagT was not stable. Since it has been established that CagX and CagT is surface exposed protein [3–4,20] therefore we wanted to see whether CagX or CagT affecting surface localization of each other or not. Few *in vivo* experiments are also required to establish these facts. We are also trying to find out the amino acid residue(s) responsible for this interaction. To find out the role of other region of CagX is also a matter of investigation. The biological significance of these new interactions may pave the way for the development of new avenues of research in TFSS. Therefore, further work is required to define the role of other component of *H. pylori* TFSS to understand these specialized and well adapted functions in detail.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.11.041.

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