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Multipronged regulatory functions of a novel endonuclease (TieA) from *Helicobacter pylori*

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ABSTRACT

Helicobacter pylori **portrays a classical paradigm of persistent bacterial infections. A well balanced homeostasis of bacterial effector functions and host responses is purported to be the key in achieving long term colonization in specific hosts. *H. pylori* nucleases have been shown to assist in natural transformation, but their role in virulence and colonization remains elusive. Therefore, it is imperative to understand the involvement of these nucleases in the pathogenesis of *H. pylori*. Here, we report the multifaceted role of a TNFR-1 interacting endonuclease A (TieA) from *H. pylori*. *tie*A expression is differentially regulated in response to environmental stress and post adherence to gastric epithelial cells. Studies with isogenic knockouts of *tie*A revealed it to be a secretory protein which translocates into the host gastric epithelial cells independent of a type IV secretion system, gets phosphorylated by DNA-PK kinase and auto-phosphorylates as serine kinase. Furthermore, TieA binds to and cleaves DNA in a non-specific manner and promotes Fas mediated apoptosis in AGS cells. Additionally, TieA induced pro-inflammatory cytokine secretion *via* activation of transcription factor AP-1 and signaled through MAP kinase pathway. Collectively, TieA with its multipronged and moonlighting functions could facilitate *H. pylori* in maintaining a balance of bacterial adaptation, and elimination by the host responses.**

INTRODUCTION

Helicobacter pylori is one of the major risk factors for the development of human gastric cancer (1). The latter is the fifth most commonly diagnosed malignancy and the third leading cause of cancer related deaths worldwide (2). *Helicobacter pylori* has coevolved with its human host for over 60 000 years (3); this extended and intimate association has fostered the emergence of various adaptive strategies that ensure persistent colonization of *H. pylori* in the gastric mucosa. Chronic infection of *H. pylori* leads to a spectrum of gastroduodenal disorders with sequelae ranging from mild superficial gastritis to duodenal ulcers, mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric adenocarcinoma (4). Nevertheless, not all infected populations are equally susceptible to develop serious clinical consequences since *H. pylori* infection is multifactorial and largely depends on the inflammatory responses mediated and sustained by host and environmental factors as well as on the activity of differentially expressed strain specific bacterial virulence proteins (strain diversity) (5,6). As much as 50% of the strain specific genes are confined to a hyper variable region known as plasticity zone (PZ) and are considered as an important source of genetic diversity (7). Several strain specific genes such as *ice*A, *ctk*A, *dup*A, *cag*A, *vac*A, *jhp0947* and others have been reported for their association with gastric diseases and inflammatory responses *via* secretion of TNF-α, IL-1β, IL-6 and IL-8 (8–13). Furthermore, *vac*A and *ctk*A induce host cell apoptosis, a balancing effect on epithelial cells turnover (9,13). Although about 20% of PZ genes are *H. pylori* specific, they lack any significant homologues available in the public databases. Functional significance of genomic diversity of *H. pylori* largelyremained obscure even after a few years of genome sequencing (14). Given that strain specific genes also contribute to colonization and pathogenicity, deciphering plausible functions of these genes is highly imperative to understand how *H. pylori* utilizes its arsenal of strain specific genes, especially given its diverse methylome (15), to adapt to the dynamic environmental conditions during long-term colonization of the host.

The 'TNFR1 interacting endonuclease A' (TieA) is a component of plasticity region (16,17), where some of the open reading frames (ORF) often encode restriction-modification (R-M) genes. Interestingly, these R-M genes also account for transcriptional regulation of other genes similar to the epigenetic mechanisms of mammalian cells (15). Notably, TieA encoded by ORF HP0986 (strain 26695) has no homolog corresponding to a known function in the available microbial sequence databases but harbors an endonuclease domain in its amino acid sequence. Epi-

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demiological studies have demonstrated significant prevalence of *tie*A gene in virtually all gastritis, peptic ulcer and gastric carcinoma associated isolates (16). *tie*A gene is expressed by the clinical isolates of *H. pylori* as well as in the *H. pylori*-positive human gastric biopsies (17). It is highly immunogenic antigen in humans and has the potential of cytoplasmic-nuclear shuttling in gastric epithelial cells (17). Moreover, purified TieA interacts with TNFR1 receptor and elicits the secretion of IL-8, IL-6 and TNF- α from macrophages by activating NF-κB driven cytokine secretion (16,18). Additionally, TieA also induces apoptosis of cultured human macrophages although the underlying signaling/molecular mechanism remains to be elucidated.

Among the various effector proteins and enzymes that are implicated in the regulation of apoptosis, deoxyribonuclease I (DNase I) has been reported to be the first enzyme identified in mammalian cells (19). It is an endonuclease, and, apart from its expression in mammalian cells, many pathogenic bacteria also produce extracellular DNases (20). These bacterial DNases/endonucleases are emerging as pathogenic traits which support bacterial growth, affect host cell pathology by enriching the pool of available nucleotides and facilitate bacterial dissemination by liquefying pus (21,22). Furthermore, extracellular DNases assist the bacteria to escape innate immune responses as they degrade neutrophil extracellular traps (NETs) (23). As stated earlier, eukaryotic DNases are known for introducing double stranded breaks in host cell DNA and are also involved in cellular apoptosis (19,24). However, it remains poorly understood as to what are the exact consequences of bacterial directed endonuclease activity within the realm of host epithelial cells. Particularly, their role in microbial growth and physiology during chronic infections remains poorly understood. The constant presence of nucleases may prove detrimental to cells and may play a more direct role in disease advancement by influencing the genetic integrity of individual host cells (25).

In this study, we identified hitherto unknown functions of TieA, especially those related to the regulation and expression of other major virulence factors such as CagA and VacA. The mRNA expression of *tie*A was differentially regulated in response to high salt concentration, low pH and adhesion to gastric epithelial cells. Further, we showed that TieA binds to and cleaves both linear and circular DNA in a sequence independent manner and functions as an endonuclease. This led us to investigate the role of TieA in induction of apoptosis. We observed that TieA translocates into the AGS cells independent of type IV secretory system (T4SS) and acts as an endonuclease protein that can single-handedly induce inflammation and apoptosis in gastric epithelial cells. These pleiotropic effects of a plasticity region protein clearly point towards the moonlighting function of TieA. Collectively, this study provides mechanistic details of the functioning of TieA, a secreted bacterial endonuclease and its contribution to virulence/colonization potential of the bacterium.

MATERIALS AND METHODS

Purification of TieA and polyclonal anti-TieA serum generation

TieA protein was purified as described earlier (16). Purified TieA was concentrated and dialyzed against 1× phosphate-buffered saline (PBS, pH 7.5) using Amicon 10 kDa filter (Millipore, USA) at 4°C. TieA was treated with polymyxin B (Sigma-Aldrich, USA) for 4 h at 4°C to eliminate the possible endotoxin contamination and was quantitated by Limulus amebocyte lysate assay using Chromogenic Endotoxin Quantitation Kit (Pierce Thermo Scientific, USA) with a sensitivity limit of 0.1 UE/ml. The protein concentration was estimated with a BCA protein assay kit (Pierce Thermo Scientific) as per the manufacturer's protocol.

Polyclonal antibodies against TieA protein were raised in Swiss albino mice. Briefly, 100 μg of purified TieA protein was mixed with equal volume of complete Freund's adjuvant (Santa Cruz, USA). This emulsified mixture was injected into the mice intradermal as a prime dose. First intradermal booster dose of TieA (200 μg) was mixed with equal volume of incomplete Freund's adjuvant and given 15 days after the prime dose. Second and third intra-dermal doses of TieA (200 μg) were given at an interval of 14 days after the first dose. After primary and secondary booster doses, mice were sacrificed, serum was collected from blood following centrifugation at 10 000 rpm and stored at −20°C. The specificity of antibody was determined by western blot analysis (Supplementary Figure S1).

Oligonucleotides and their radiolabeling

All the oligos used by us were synthesized by Bioserve Pvt. Ltd. (Hyderabad, India) (Supplementary Table ST1). The oligonucleotides were annealed to form duplex DNA by mixing the equimolar amount of two oligonucleotides (diluted to 250 μg/ml) in an annealing buffer comprising of 10 μl of oligos A and B, 5 μl of 10× T4 polynucleotide kinase buffer and 25 μl of ultra-pure water (Invitrogen Life Technologies, USA). The mixture was heated to 65°C for 5 min and allowed to cool slowly to room temperature. The annealed oligonucleotides were labeled at the 5′ end with [γ^{32}-P] ATP (BRIT, India) using T4 polynucleotide kinase (Sigma-Aldrich) and purified with microspin-G25 columns (GE Healthcare, Sweden). Labelling of oligonucleotides was carried out as follows: 1 μl of annealed DNA oligos were mixed with 5 μl of 10× T4 polynucleotide kinase buffer, 2.5 μl [γ^{32}-P] ATP (1mCi), 0.5 μl of 0.5 M DTT, 1 μl of dNTPs and 1 μl of T4 polynucleotide kinase.

Electrophoretic mobility shift assay (EMSA)

For DNA binding reactions, TieA was pre-incubated in binding buffer containing 20 mM HEPES (pH 7.9), 1 mM DTT, 0.1 mM EDTA, 50 mM KCl, 5% glycerol and 200 μg/ml BSA (bovine serum albumin). For competition experiments, excess of unlabeled competitor was carried in the reaction mixture and Poly d[I-C] (Sigma-Aldrich) was used as a competitor DNA. After 30 min of incubation at 4°C, 1 nM labeled DNA (^{32}P-dsDNA) substrate was added. The

reaction was carried out for another 20 min at 37 °C before the samples were loaded on 6% non-denaturing native polyacrylamide gel in 45 mM Tris/borate (pH 8.3) containing 0.4 mM EDTA (0.5× TBE) buffer to separate protein–DNA complexes from free DNA. The gel was transferred to a Whatman paper, dried under vacuum at 75°C for 30 min and exposed on an autoradiography film overnight prior to visualization by phosphorimaging.

Nuclease assay

Nuclease assay was performed in a 30 μl reaction mixture containing 1 μg of pUC19 or lambda DNA substrate (New England Biolabs). Briefly, 1 μg of the DNA substrate was incubated with indicated concentration of TieA protein in 1× NEB cut smart buffer (New England Biolabs). The cleavage reaction was initiated with the addition of MboII or DNase I (1 U/reaction, New England Biolabs) enzyme. Digestion was carried out for 1 h at 37°C and reaction was terminated by adding 10 mM EDTA. The samples were deproteinized due to proteinase K (10 μg/reaction) in the presence of 0.05% SDS for 15 min at 65°C. Samples pre-incubated with 10 mM EDTA and 0.05% SDS were used as a negative control. The digested products were separated on 1.2% agarose gel and run in 1× TAE buffer.

H. pylori culture

Isogenic knockout of CagPAI (26695Δ*cag*PAI) was a gift from Wolfgang Fischer (Max von Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie) Munich, Germany. CagE (26695Δ*cag*E) and CagA (26695Δ*cag*A) mutants were gifts from G. Mukhopadhyay, JNU, New Delhi. *H. pylori* strains were grown as described previously (26). Wild type strains (P12, 26695) were cultivated on GC agar plates containing horse serum supplemented with vancomycin (10 μg/ml), trimethoprim (2.5 μg/ml) and nystatin (2 μg/ml). Mutants 26695Δ*cag*A and 26695Δ*cag*E were cultivated on selective chloramphenicol (4 μg/ml) GC agar plates. P12Δ*tie*A and 26695Δ*cag*PAI were cultivated on selective kanamycin (10 μg/ml) GC agar plates. In addition, for *H. pylori* liquid cultures, Brain Heart Infusion (BHI) (BD Difco) medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen Life Technologies) was used; the medium was inoculated with a bacterial suspension with an optical density of 0.1 at 550 nm. In some experiments, the salt concentration of the BHI-FBS was altered by adding 1.25% NaCl (215 mmol/l). In another set of experiments, the pH of the medium (BHI–FBS) was adjusted with the help of hydrochloric acid before the addition of *H. pylori*. The cultures were further incubated in jars (Borosil, India) containing Gaspak EZ anaerobe (BD Biosciences, USA) for 14 h (OD_{550}: 0.5). The 14 h liquid cultures of P12 and 26695 strains (OD_{550}: 0.5) were harvested and to ensure the viability of the *H. pylori* strains at pH 4.0, aliquots were collected from the media after every 3 h of pH exposure and plated on GC agar plates to determine the number of colony forming units (CFU).

TCA precipitation

The exponentially growing *H. pylori* cultures (14 h) were centrifuged at 10 000×g for 10 min. Supernatant was collected and filtered through 0.45 μm pore size filter (Millipore). 400 μl of 50% trichloroacetic acid (TCA) was added into 1 ml of filtered supernatant and incubated at 4°C for 1 h. After incubation, the supernatant was centrifuged at 14 000 rpm for 15 min and the obtained pellet was washed twice with ice cold acetone (200 μl). The pellet was further centrifuged at 14 000 rpm for 5 min, dried at 95°C for 5 min and was finally resuspended in 100 μl of 2× Laemmli buffer.

Generation of isogenic *tie*A mutants

*tie*A mutants were constructed in *H. pylori* strains 26695 and P12 by homologous recombination method. Briefly, 500 bp upstream and 500 bp downstream of *tie*A was amplified by polymerase chain reaction with 18 nucleotide flanking sequences as primers. The resulting PCR products were digested with BamHI (New England Biolabs) and ligated subsequently. The fragments were cloned at Xho I–Bam HI sites of the pBluescript vector and separated by an *aph*A gene cassette. The resulting construct (50 μg) was transformed naturally into *H. pylori.* The mutants were selected by plating on selective GC agar plates. DNA was extracted from the colonies obtained and further analyzed by qRT-PCR and western blot analysis (Supplementary Figure S2). A single recombinant clone for each mutant was used for infection experiments.

Cell lines, culture and infection experiments

The human monocyte/macrophage (THP-1) and human gastric adenocarcinoma cell lines (AGS) were procured from National Centre for Cell Science, Pune, India. AGS and THP-1 cell lines were grown in Ham's-F12 and RPMI 1640 media (Invitrogen Life Technologies), respectively, after supplementing with 10% (vol/vol) heat inactivated fetal bovine serum (FBS) (Invitrogen Life Technologies) and 1% antibiotic–anti-mycotic solution (Invitrogen Life Technologies). Cells were maintained in a humidified incubator with 5% CO_2 at 37°C. THP-1 cells were allowed to differentiate into macrophages with the treatment of 10 ng/ml phorbol myristate acetate (PMA) (Sigma-Aldrich) for 48 h. After 48 h, RPMI 1640 medium was aspirated, replaced with fresh medium with 10% FBS and maintained for another 24 h. For infection experiments, cells were seeded in 6 well plates or in 100 mm dishes (Corning, USA) (70% confluent). Cells were serum starved for 16 h before infection and were later infected with *H. pylori* at a multiplicity of infection (MOI) of 100.

TieA translocation studies

AGS cells were infected with P12, P12Δ*tie*A and 26695Δ*cag*PAI strains of *H. pylori* for 8 h, and incubated in a humidified incubator with 5% CO_2 at 37°C. After infection, non-adherent *H. pylori* were removed by washing (three times) with ice-cold 1× phosphate buffer saline (PBS, pH 7.5) containing 100 mM sodium vanadate (Invitrogen Life Technologies). Cells were processed as

described earlier with minor modifications (27). Briefly, infected AGS cells were scraped in 2 ml of 1× PBS/sodium vanadate. Cells from four 100 mm dishes were combined and pelleted down at 200×g for 5 min. Then, the supernatant was discarded and cell pellet resuspended in 8 ml of homogenization buffer (250 mM sucrose, 3 mM imidazole (pH 7.4), 0.5 mM EDTA). Next, the cells were allowed to pellet for 10 min at 1500×g followed by resuspension in 600 μl of the same buffer. The resuspended cells were mechanically lysed by 1 ml syringe carrying 0.22 gauge needle by expelling aggressively (four times). The lysed cells were then pelleted for 10 min at 41 000×g and cell pellet containing bacteria, unlysed cells was resuspended in 600 μl 2× Laemmli buffer. The supernatant containing host cell cytosol and membrane fractions was mixed with 4× Laemmli buffer. The samples were boiled at 95°C for 10 min and were assessed by separating on 12.5% SDS-PAGE. Separated proteins were transferred to a PVDF membrane and TieA translocation was detected using anti-TieA antibody.

In vitro kinase assay

Recombinant purified TieA (1 μg) was incubated with CKII (1 unit), 200 μM ATP, 1 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) in 1× NEB buffer (New England Biolabs) at 30°C for 30 min. The reaction was terminated by addition of 4× Laemmli SDS sample dilution buffer. Proteins were separated by 12.5% SDS-PAGE and phosphorylation was visualized by western blot (by using monoclonal Ser/Thr antibody (Sigma-Aldrich).

For DNA-PK kinase assay, purified recombinant TieA (1 μg) was incubated in 2× assay buffer (2 mM DTT, 2 mM $MnCl_2$, Na_3Vo_4), 250 μM ATP and DNA-PK activation buffer which contains calf DNA and DNA-PK kinase. The reaction mixture was incubated at 30°C for 15 min as described elsewhere (9). DNA-PK substrate and DNA-PK kinase were replaced by TieA in the test reaction for autophosphorylation. Finally, 25 μl ADP-Glo reagent was added to deplete remaining unused ATP and plate was incubated at room temperature for another 40 min with constant shaking. Afterwards, 50 μl kinase detection reagent was added and allowed to incubate for 30 min at room temperature prior to read the plate using a luminometer.

Measurement of cell death and Fas expression

Cell death was determined by measuring cytoplasmic histone-associated DNA fragments (28). Briefly, AGS cells were seeded in 96-well plate and infected with both wild type (WT) (P12) and *tie*A knock out (KO) *H. pylori* (P12Δ*tie*A) at an MOI of 100. After 24 h of infection, cell death was estimated with the cell death detection ELISA Plus kit (Roche, USA) according to the manufacturer's method. For measurement of Annexin V-FITC binding, infected AGS cells were processed and stained with FITC conjugated Annexin V as per the manufacturer's protocol (BD Biosciences). To determine necrosis, 5 μl of propidium iodide (PI) (100 μg/ml) was added to the cell suspension 10 min before analysis and stained AGS cells were acquired on flow cytometer (BD FACS canto II). The percentage of cells that underwent apoptosis was expressed as total number of % Annexin V^+ and PI^+ cells after subtracting background fluorescence. Un-infected cells were used as negative control and staurosporine (50 nM, 3 h of treatment) treated cells served as a positive control. Fas expression was determined as described elsewhere (19).

DNA ladder assay

AGS cells (4×10^6) were harvested by trypsin digestion and fixed with 4% paraformaldehyde for 20 min at room temperature. The fixed cells were permeabilized with 0.5% Triton X-100 for 30 min at room temperature, followed by incubation with 5 μg TieA for 16 h at 37°C. After 16 h, chromosomal DNA was isolated as described elsewhere (18). The extracted DNA was analyzed by 1.5% agarose gel electrophoresis and stained with ethidium bromide.

RNA isolation, expression analysis and RT^2 profiler array

Total RNA was isolated from *H. pylori* strains grown under high NaCl concentration and low pH using Trizol reagent (Invitrogen Life Technologies) according to the manufacturer's protocol. RNA samples (1 μg) were subjected to RNase free DNase digestion by using amplification grade DNase I (Sigma-Aldrich) enzyme. cDNA synthesis was performed with 1 μg of purified RNA using the superscript III cDNA synthesis kit (Invitrogen Life Technologies). First strand cDNA reactions were performed as control, in parallel, without adding reverse transcriptase. Quantitative real time PCR was performed in triplicate on a Mastercycler® *epgradient* realplex^4 PCR machine (Eppendorf, Germany) by using the primers mentioned in supplementary Table ST2. Fold change in *cag*A, *vac*A and *tie*A expression was calculated by ΔΔCT method, with each transcript level normalized to the 16S rRNA/ureA internal control and compared to the normalized transcript levels of WT *H. pylori*. In another set, a human antibacterial response RT^2 profile PCR array was carried out according to manufacturer's instructions (Qiagen, Germany). Total RNA was prepared from AGS cells infected with *H. pylori* and *tie*A KO *H. pylori* as described above. RNA samples (1 μg) were transcribed into cDNA by using RT^2 first strand synthesis kit (Qiagen). Next, the cDNA was mixed with RT^2 qRT PCR mix (Qiagen) supplied by the manufacturer and real time PCR was set up in a 96-well plate format. Expression of 84 genes involved in innate immune response was examined by using RT^2 profiler PCR array kit (Human antibacterial response PHAS 148Z). Fold change and expression analysis were computed by manufacturer's online analysis tool. Gene expression was normalized to housekeeping genes.

Adhesion assay

AGS cells were infected with *H. pylori* for 6 h at an MOI of 100. Infected cells were washed three times with 1X PBS to remove the unadhered bacteria and lysed with 1 ml of PBS containing 0.1% Triton X-100 by vigorous pipetting. The lysate was diluted in Brain Heart Infusion (BHI) medium in a final volume of 1 ml. Twenty microliter of each dilution was plated on GC agar plates and incubated at 37°C in a

humidified microaerophilic environment containing 5% O_2 and 5% CO_2 for 48 h. The adhered *H. pylori* were enumerated as CFU.

Western blotting

SDS-PAGE, transfer of TieA to PVDF membranes and its probing with primary and secondary antibodies were carried out as per standard procedures. For TieA detection, anti-TieA was used at 1:5000 dilutions in 1× PBST (1× PBS + 0.05% Tween 20) for 3 h at room temperature. phospho-ERK, phospho-JNK and phospho-p38 antibodies (eBiosciences, USA) were used at 1:500 dilution in 1× TBST for 3 h. Anti-CagA (Santa Cruz) was used at 1:1000 dilution in 1× TBST. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies (Sigma-Aldrich) were used at 1:80 000 dilutions. Development of the blots was done using SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Scientific).

Detection of MAPK phosphorylation and ELISA

Activated phospho-ERK, phospho-p38 and phospho-JNK were determined by instant one ELISA kit (eBiosciences) as per the manufacturer's instructions. Briefly, after 1 h post-infection, Ham's-F12 medium was removed and cells were washed with 1× PBS. After washing, cells were lysed with 300 μl of freshly prepared lysis buffer (provided in kit) with shaking (∼300 rpm) at room temperature for 10 min. 50 μl of the prepared sample lysate was incubated with 50 μl of antibody cocktail (capture and detection antibody) for 1 h at room temperature. After incubation, cells were washed three times with 1× wash buffer and 100 μl of detection reagent was added into the wells for 20 min at room temperature in dark. 100 μl of stop solution was added into each well and absorbance of the samples was measured using Tecan ELISA plate reader at 450 nm. For p38 and JNK inhibition experiments; AGS cells were pretreated with SB203508 (p38 inhibitor) and SP600125 (JNK inhibitor) at the indicated final concentration (both from Sigma-Aldrich) for 1 h prior to TieA treatment. As a negative control, AGS cells were treated with dimethyl sulfoxide alone. For cytokine analyses (IL-8, IL-6, IL-1β and TNF-α), ready set Go ELISA kit (eBiosciences) was used as per the manufacturer's protocol.

Statistics

Statistical analyses were performed with GraphPad Prism 5.0 software. Relative expression of *tie*A was compared by one-tailed Student's *t* test. The phospho-ERK, phospho-p38, phospho-JNK and phospho c-Jun levels were compared by two-tailed Student's *t* test. *P* values of <0.05 were considered as statistically significant.

RESULTS

Prevalence of *tie*A in sequenced *H. pylori* genomes

We analyzed the prevalence of *tie*A among various complete and draft *H. pylori* genomes available at NCBI database using BLASTn. Out of 52 complete genomes, *tie*A was present in 17 genomes accounting for ∼33% prevalence. The scenario was not very different in the case of 413 draft genomes analyzed wherein, *tie*A was found to be present in 138 genomes (∼32%) (Figure 1A). Taking into account the different lineages of *H. pylori*, *tie*A gene was mostly prevalent in strains belonging to hpEurope, hpSouthIndia and hspAmerind lineages while being completely absent in East Asian strains.

*tie*A expression is upregulated in response to high NaCl and low pH

A remarkable expertise of *H. pylori* lies in its ability to survive in harsh environment of stomach, where it encounters osmotic and pH stresses (29,30). Survival of *H. pylori* requires adaptation which could be achieved through regulation of bacterial gene expression thereby allowing the bacterium to acclimatize to the dynamic niches. Hence, we analyzed the mRNA levels of *tie*A in response to varying NaCl concentration and low pH. *H. pylori* strains 26695 and P12 were grown in BHI media containing two different salt concentrations (BHI-FBS-0.5% NaCl and BHI-FBS-1.25% NaCl) and transcript levels were compared. The mRNA level for *tie*A was significantly upregulated by 2.5-fold and 5-fold in 26695 and P12, respectively, when grown in BHI-FBS-1.25% NaCl as compared to *H. pylori* grown in BHI-FBS-0.5% NaCl (Figure 1B). Additionally, exposure of *H. pylori* to pH 4.0 resulted in >5-fold (26695) and 15-fold (P12) upregulation in the mRNA levels of *tie*A relative to *H. pylori* grown in BHI medium at pH 7.0 (Figure 1C). Furthermore, to reveal the effect of low pH exposure on *H. pylori* strains, it was essential to ascertain that strains would be viable during the course of experiment. Growth curve analysis and number of cfu/mL illustrated survival of the *H. pylori* strains throughout the course of experiment (Supplementary Figure S3). These results imply that *tie*A transcription is potentially upregulated in response to high salt and low pH stress.

*tie*A expression elevates upon *H. pylori*'s adherence to gastric epithelial cells

H. pylori is known to alter the expression of different sets of virulence genes such as *cag*A and *ice*A in response to adherence to gastric epithelial cells (31,32). Hence, we sought to examine the mRNA levels of *tie*A in *H. pylori* strains P12 and 26695 post adherence to AGS cells. A significant upregulation of *tie*A mRNA transcript levels of >5-folds for P12 and 26695 strains was observed at 6 h post infection as compared to *H. pylori* cultured under similar conditions without AGS cells (Figure 1D). Thus, we next examined whether lack of *tie*A affects the adherence of *H. pylori* to AGS cells. In order to delineate this, AGS cells were infected with WT and *tie*A KO *H. pylori* for 6 h and adherence capability was determined by measuring cfu/mL. No significant difference was observed for cfu/mL between WT and *tie*A KO *H. pylori*, demonstrating that lack of *tie*A did not affect the adhesion capability of *H. pylori* to AGS cells (Figure 1E). These findings suggest that *tie*A has no role in adherence of *H. pylori* to AGS cells and direct contact of *H. pylori* is required for upregulated transcript levels of *tie*A.

A

H. pylori lineages	No. of genomes	tieA status
HspEastAsia	13	0
HpEurope	15	8
HpSouthIndia	03	2
HspAmerind	14	7
HpAfrica1(wa)	4	0
HpAfrica1(sa)	3	0

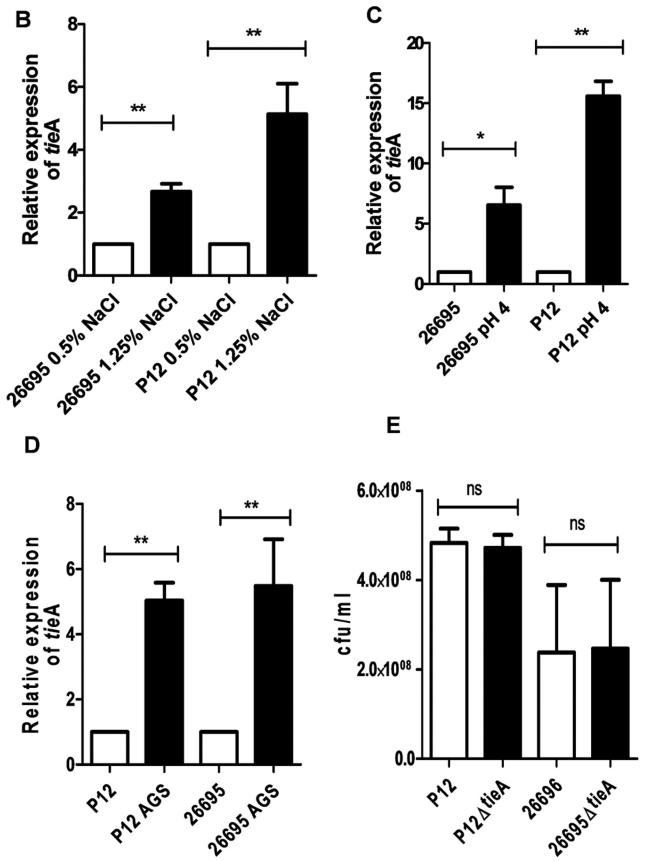

Figure 1. (**A**) Prevalence of *tie*A in *H. pylori* genomes; table represents the presence of *tie*A in complete genomes of different lineages of *H. pylori* available at NCBI database. (**B** and **C**) qRT-PCR analysis of *tie*A mRNA levels in high NaCl concentration and low pH relative to *H. pylori* strains grown in 0.5% NaCl, and pH 7.0 respectively. Relative expression was calculated with each transcript level normalized to the 16S rRNA and ureA as internal controls. Data represent the mean ± SD of three independent experiments; one-tailed Student's *t* test was performed wherein $*P \leq 0.05$, $**P \leq 0.01$. (**D**) Expression of *tie*A upon adherence to gastric epithelial cells by qRT-PCR. Total RNA was extracted from AGS cells infected with P12 or 26695 strains of *H. pylori* for 6 h (MOI of 100). As a control, *tie*A expression was estimated in *H. pylori* strains incubated under identical conditions without AGS cell lines. 16S rRNA and *ure*A were used as endogenous controls for normalization. Data represent the mean±SD of three independent experiments; one-tailed student's *t*-test was performed wherein $*P \leq 0.05$, $**P \leq 0.01$. (**E**) The adherence capability of WT and *tie*A KO strains on AGS cells was estimated as cfu/mL. Data represent the mean±SD of three independent experiments; one-tailed Student's *t* test was performed wherein $*P \leq 0.05$, $**P \leq 0.01$.

*cag*A and *vac*A expression upregulates in *tie*A deficient strains of *H. pylori*

The mRNA transcript levels of *cag*A and *vac*A were evaluated in *tie*A KO *H. pylori*. Intriguingly, the expression levels of *cag*A were significantly upregulated by 6.5-fold in 26695Δ*tie*A and by 3-fold in P12Δ*tie*A *H. pylori* (Figure 2A). The mRNA levels of *vac*A were also upregulated by >15-fold in both 26695Δ*tie*A and P12Δ*tie*A strains of *H. pylori* (Figure 2B). Comparably, we next investigated whether *cag*A deficient *H. pylori* alter the mRNA level of *tie*A in a way similar to *tie*A KO *H. pylori*. The mRNA levels of *tie*A were also upregulated by 4.5-fold in *cag*A deficient strain (Figure 2C). To eliminate any possibility of non-specific effects of deletion of *tie*A, we examined whether absence of *cag*E also alters the mRNA expression of *tie*A in *cag*E KO *H. pylori.* Notably, the mRNA levels of *tie*A in *cag*E KO *H. pylori* remained unaltered (Figure 2C). Following the above lead, we next analyzed whether lack of *tie*A influenced the morphology of AGS cells *in vitro*. To address this possibility, AGS cells were infected with WT, *tie*A KO and *cag*A KO *H. pylori* at an MOI of 100 for 6 h. AGS cells infected with *tie*A KO *H. pylori* showed almost similar extent of humming bird phenotype as compared to WT *H. pylori* (Figure 2D), whereas AGS cells infected with *cag*A KO *H. pylori* did not induce humming bird formation. Interestingly, *tie*A KO *H. pylori* showed an upregulated *cag*A mRNA expression, however, alteration of *tie*A mRNA transcripts did not correlate with the observed differences in the induction of humming bird phenotype in AGS cells. These results suggest that absence of *tie*A alters the *cag*A transcript levels but did not affect humming bird formation phenotypically.

TieA binds to DNA non-specifically and cleaves naked DNA in a Ca^{2+}- Mg^{2+} dependent manner

After evaluating the expression analysis of *tie*A in response to different environmental stimuli, we next aimed to identify the functional domains present in TieA protein sequence by InterPro (33). The analysis revealed presence of a type II endonuclease domain, tRNA endonuclease domain and a domain of unknown function (DUF) in TieA protein sequence. To confirm the putative endonuclease activity of

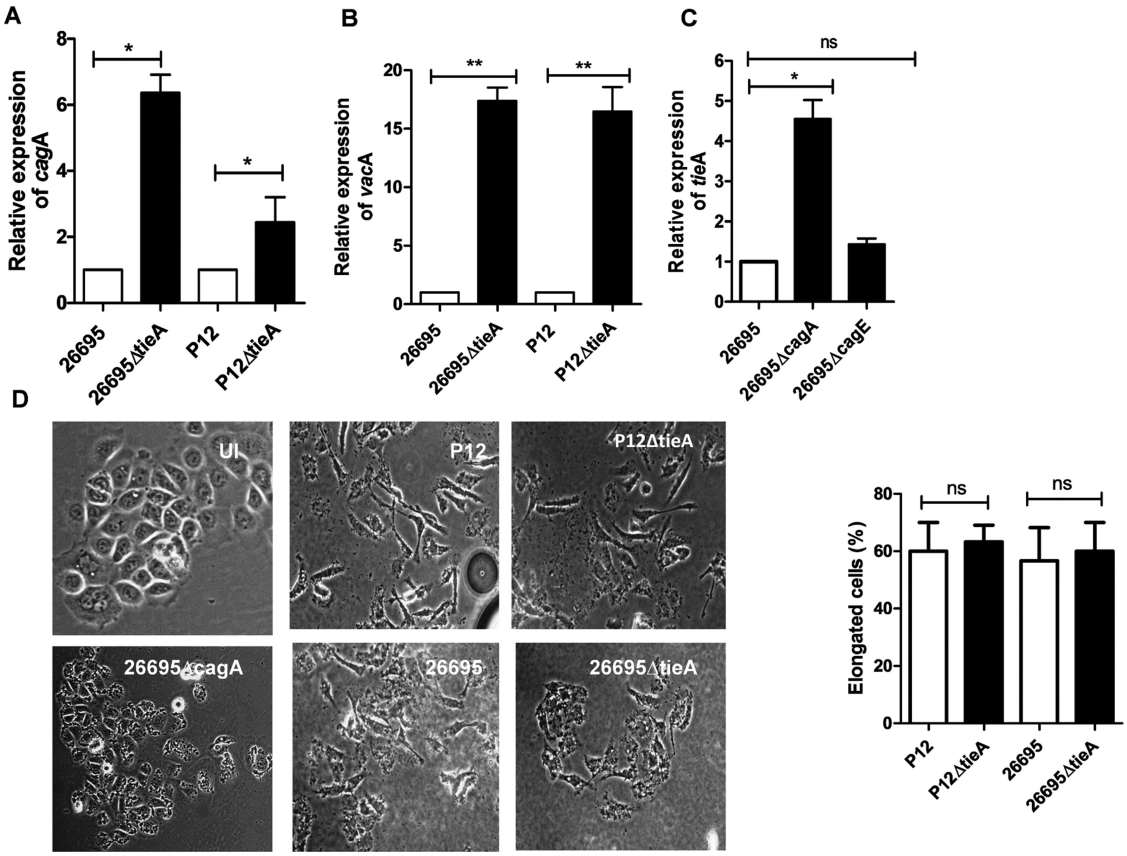

Figure 2. (**A**) *cag*A mRNA expression levels in *tie*A KO *H. pylori* relative to 26695 and P12 *H. pylori*. (**B**) *vac*A mRNA expression levels in *tie*A KO *H. pylori* relative to the 26695 and P12 strain. (**C**) *tie*A mRNA levels in *cag*A and *cag*E KO *H. pylori*: mRNA levels of *tie*A were upregulated in *cag*A KO strain but remained unchanged in *cag*E KO strain. (**D**) Humming bird phenotype formation in AGS cells following infection with WT *H. pylori, tie*A KO and *cag*A KO *H. pylori* at MOI of 100 for 6 h. AGS cells were examined by light microscopy at 20× magnification. The number of elongated cells were counted and expressed as percentage of total cells in each sample (bar diagram). Data shown as mean ± SD of three independent experiments; one-tailed Student's *t* test was applied, $*P \leq 0.05$, $**P \leq 0.01$, ns: non-significant.

TieA, DNA binding activity of TieA was examined initially by EMSA. Different concentrations of protein at 0.1, 0.5, 1 and 2 μg, were used as indicated in figure 3A. It was observed that TieA binds to dsDNA from 0.5 μg of protein concentration onwards resulting in retardation of DNA–protein complex on native PAGE (Figure 3A). To determine whether TieA-DNA binding is sequence specific or independent, EMSA was carried out with mutated oligos. TieA showed a similar binding pattern as observed with non-mutated oligos (Figure 3B). These results demonstrated that TieA binds to DNA in a non-specific manner. We also tested the ability of purified TieA to cleave plasmid DNA by *in vitro* nuclease assay. The lambda (linear) and pUC19 plasmid DNAs (circular) were used as substrates. DNase I and MboII enzymes were used as positive controls and, Rv3131 (an unrelated recombinant protein purified in a similar manner) was used as a negative control. TieA showed a non-specific endonuclease activity similar to DNase I as it cleaved both circular pUC19 and linearized lambda DNA, whereas Rv3131 (lane 6) as well as heat-inactivated TieA (lane 4) did not show any detectable DNA cleavage activity (Figure 3C). Furthermore, our results demonstrated a Ca^{2+}–Mg^{2+}dependent DNA cleavage activity of TieA, since EDTA and SDS markedly inhibited the cleavage activity of TieA (Figure 3D, lanes 8, 9, 17, 18). These findings together indicated that the TieA binds to and cleaves DNA non-specifically in a Ca^{2+}–Mg^{2+}dependent manner.

TieA degrades chromosomal DNA in prefixed AGS cells

As a follow-up of the above results, we investigated if TieA is also capable of digesting chromosomal DNA. To determine this, we examined the induction of DNA fragmentation of prefixed AGS cells upon treatment with different concentrations of TieA. Our results revealed a nucleosome pattern of DNA degradation in prefixed AGS cells treated with TieA (Figure 4A). A similar pattern of chromosomal DNA degradation was also observed when the prefixed AGS cells were incubated with staurosporine (50 nM), a well-known inducer of apoptosis. However, the heat inactivated TieA did not degrade the chromosomal DNA in prefixed AGS cells and gave similar pattern as obtained for untreated AGS cells (Figure 4A). Degradation of nuclear DNA is dependent upon the activity of the endogenous DNases. But in normal living or prefixed cells, endogenous DNases cannot access the chromosomal DNA (34). Therefore, the probability of degradation of chromosomal DNA by endogenous DNases in prefixed cells is negligible. These results demon-

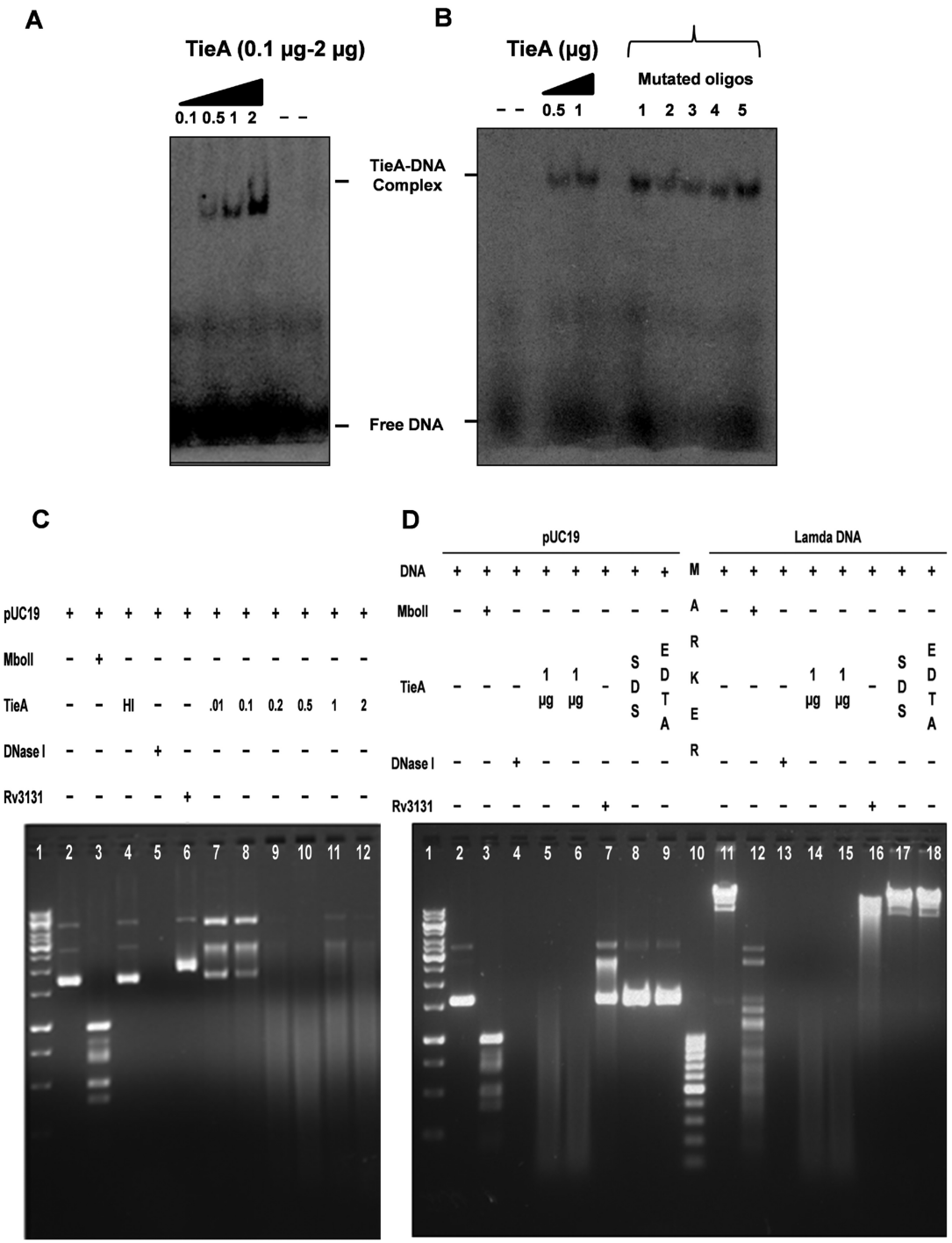

Figure 3. (**A**) Binding of TieA to dsDNA: electrophoretic mobility shift assays were carried out by incubating different concentrations of TieA (0.1, 0.5, 1 and 2 µg) with 0.5 nM ^{32}P-labeled DNA substrates. Samples were subjected to electrophoresis on native PAGE and visualized by autoradiography as mentioned in materials and methods section. (**B**) TieA binds to DNA non-specifically: electrophoretic mobility shift assays were carried out by incubating 1 µg of TieA with mutated oligos 1–5 (see Supplementary Table S1). (**C**) Nuclease activity of TieA: different concentrations of TieA (0.01, 0.1, 0.2, 0.5, 1 and 2 µg corresponding to lanes 7-12, respectively) were incubated with 1 µg of pUC19 DNA for 1 h at 37 °C. The reaction was stopped by addition of 10 mM EDTA and samples were deprotonized by adding proteinase K (10 µg/sample) in presence of 0.05% SDS for 15 min at 65°C. The digested products were separated on 1.2% agarose gel. Rv3131 (0.5 µg) was used as a negative control in lane 6. MboII (1 unit/reaction) and DNase I (1 unit/reaction) served as positive controls in lanes 3 and 5, respectively. Lane 4 represents heat inactivated TieA. (**D**) TieA cleaves both pUC19 (circular) and Lambda DNA (linear): pUC19 and Lambda DNA were incubated with TieA (lanes 5, 6, 14 and 15) for 1 h at 37°C and processed as described above. MboII (lanes 3 and 12) and DNase I (lanes 4 and 13) were used as positive controls. Rv3131 protein was used as a negative control (lanes 7 and 16). Ca^{2+}–Mg^{2+} dependent nuclease activity of TieA was confirmed by pre-incubating pUC19/Lambda DNA with either SDS (0.05%) or EDTA (10 mM) for 10 min (lanes 8, 9, 17 and 18) and later 1 µg of TieA was added and further processed as described above. Data are representative of three independent experiments. HI: heat inactivated.

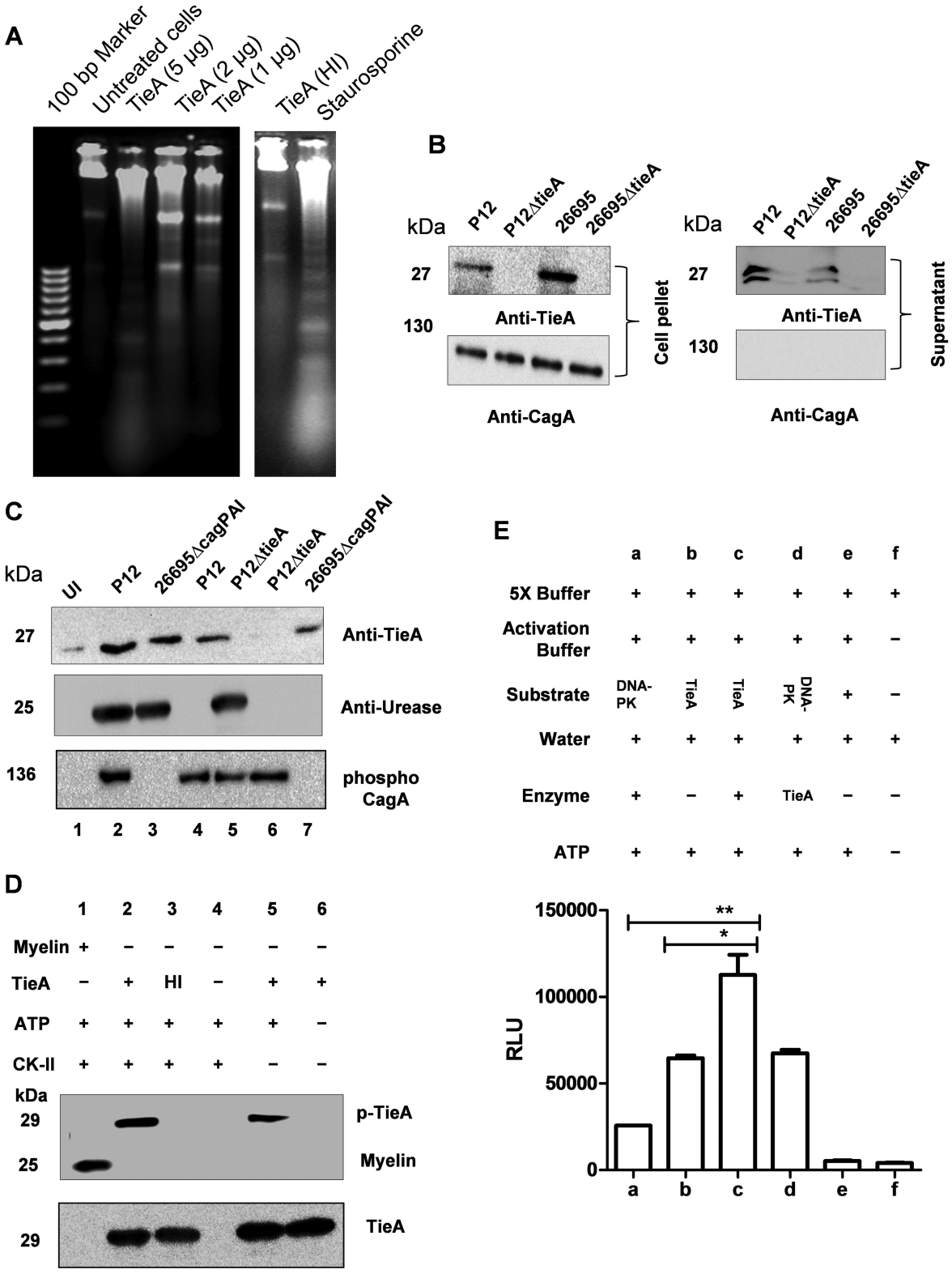

Figure 4. (**A**) DNA fragmentation of AGS cells by ladder assay: the prefixed and permeabilized AGS cells were incubated with different concentrations of TieA (1, 2 and 5 µg) for 16 h at 37°C. Untreated cells were used as negative control and staurosporine treated cells were used as positive control. (**B**) Immunoblot analysis for TieA protein secretion: *H. pylori* strains P12 and 26695 were grown in BHI broth with 10% FBS and presence of TieA and CagA was determined by TCA precipitation in pellet and supernatant fractions of BHI broth using anti-TieA and anti-CagA antibodies. Pellet and supernatant fractions showed presence of TieA protein whereas CagA was present only in pellet of *H. pylori.* (**C**) TieA translocates into the AGS cells: AGS cells were infected with both WT and *tie*A KO *H. pylori* at an MOI of 100 for 8 h as described in Materials and Methods. Host cell cytosol and membrane fractions

strated that TieA mediated chromosomal DNA degradation of AGS cells was independent of the activity of endogenous DNases such as EndoG and CAD/DFF40.

TieA is secreted in culture supernatant of *H. pylori*

Consistent with the idea that numerous pathogenic bacteria produce and secrete endonucleases or DNases in the extracellular environment (20), we sought to assess the release of TieA in *H. pylori* culture supernatant. *H. pylori* strains P12 and 26695 were cultured in BHI broth for 14 h and fractionated by TCA precipitation. The supernatant and the lysate fractions were analyzed by immunoblotting with anti-TieA polyclonal as well as by anti-CagA antibodies. The type IV secretion effector protein CagA was used as a control; since direct host cell contact is indispensable for the secretion of CagA, thereby excluding the possibility of artificial lysis of *H. pylori* during the experimental procedure. Immunoblot analysis showed the presence of TieA in both pellet and supernatant of the *H. pylori* liquid culture (Figure 4B). However, T4SS effector protein CagA was present in the *H. pylori* cell pellet but absent in the corresponding supernatant fraction (Figure 4B). These data demonstrated that TieA is a secretory protein.

TieA translocates inside the host cells independent of type IV secretory system

We next deciphered the fate of secreted TieA by determining whether the protein remains extracellular or translocates inside the host cells. To address this, AGS cells were infected with P12, P12Δ*tie*A and 26695Δ*cag*PAI *H. pylori* for 8 h, followed by examination of cytosol/membrane fractions of infected AGS cells for the presence of TieA. To confirm that the lysis of *H. pylori* should not occur during cell fractionation, we employed urease as a negative control and CagA as a positive control. Urease is a bacterial cytosolic protein that does not translocate inside the host cells. Therefore, urease could not be identified in the host cell cytosol or membrane fraction (Figure 4C). Immunoblot analysis with anti-TieA polyclonal antibody demonstrated the presence of TieA in the membrane/cytosol fractions of P12 and 26695Δ*cag*PAI infected AGS cells (Figure 4C). But it was completely absent in the membrane fractions of P12Δ*tie*A infected AGS cells. The translocation of TieA remained unaffected in the case of AGS cells infected with *cag*PAI deleted *H. pylori* which are deficient in T4SS apparatus. These results illustrated that TieA translocated inside the AGS cells independent of type IV secretion system.

TieA is capable of autophosphorylation as serine kinase and gets phosphorylated by DNA-PK kinase

Many translocated bacterial proteins undergo phosphorylation by host kinases at tyrosine, threonine and serine residues (35). Therefore, we assessed whether TieA could also undergo phosphorylation; Netphosbac analysis (http://www.cbs.dtu.dk/services/NetPhosBac-1.0/) illustrated that TieA protein sequence has the following serine residues: AECK**S**GELK, YINN**S**RR, RKNL**S**SLLY, KNL**S**SLLYE which could be phosphorylated by the serine kinases. *In vitro* phosphorylation assay was performed to check if these residues are recognized by recombinant serine kinases to phosphorylate TieA. Myelin basic protein was employed as a general serine/threonine kinase substrate. TieA was tested for phosphorylation by a serine kinase casein kinase-II (CKII) and probed against a monoclonal phospho-serine antibody. Our results demonstrated that TieA was phosphorylated at serine residues in the presence of CKII kinase (Figure 4D). However, we also detected a band at approximately 29 kDa in reaction without CKII kinase, which corresponds to the autophosphorylated TieA (Figure 4D). As we could not negate a possible autophosphorylation activity of TieA in reaction with CKII kinase, it implies that TieA has an autophosphorylation activity. In an attempt to determine the ability of TieA to get phosphorylated by DNA-PK kinase, luminescence based kinase assay approach was employed. Our results revealed that TieA indeed gets phosphorylated in the presence as well as in the absence of DNA-PK kinase (Figure 4E). However, in the presence of DNA-PK kinase, the phosphorylation state of TieA gets enhanced as shown by relative light units (Figure 4E). Collectively, these findings demonstrated that TieA autophosphorylates as serine kinase and undergoes phosphorylation by DNA-PK kinase or TieA serves as a DNA-PK substrate *in vitro*.

*tie*A induces innate immune response in gastric epithelial cells

Secreted and translocated components of *H. pylori* are described to mediate various host-pathogen interactions during the course of an infection (36) and the manipulation or induction of innate immune responses is one such state of action (26). Therefore, the innate immune response to-

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(supernatant) were separated from the cellular fraction (pellet) containing the unlysed cells, bacteria and the cytoskeletal proteins. Urease was used as negative control and Cag A was used as a positive control. The fractions were analyzed by western blot by using anti-TieA, anti-urease and anti-tyrosine phospho antibodies (for CagA) (phospho CagA). Lane 1 – AGS cells, lane 2 – *H. pylori* (P12) + cell pellet, lane 3 – *H. pylori* (26695ΔcagPAI) + cell pellet, lane 4 – *H. pylori* (P12) + host cell membrane and cytosol, lane 5 – *H. pylori* (P12ΔtieA) + cell pellet, lane 6 – *H. pylori* (P12ΔtieA) + host cell membrane and cytosol, lane 7 – *H. pylori* (26695ΔcagPAI) + host cell membrane and cytosol. (**D**) *In vitro* kinase assay for phosphorylation of TieA by CKII enzyme as analyzed by western blot. Lane 1 represents reaction with Myelin basic protein (positive control), lane 2 represents TieA (1 μg) as a substrate for CKII enzyme, lane 3 represents kinase reaction using heat inactivated (HI) TieA, lane 4 represents a negative control, lane 5 represents the kinase reaction with TieA and ATP (autophosphorylation), lane 6 represents reaction with TieA alone. Following kinase reaction, phosphorylation of TieA was probed using anti-serine antibody. (**E**) *In vitro* kinase assay for detection of phosphorylation of TieA by DNA-PK. X axis represents different reaction conditions; lane a represents positive kinase reaction containing DNA-PK substrate (0.5 μg) and DNA-PK kinase, lane b represents reaction with TieA (1 μg) + ATP only and demonstrated auto-phosphorylation of TieA, lane c represents TieA (substrate) + DNA-PK (enzyme) + ATP and demonstrated autophosphorylation as well as phosphorylation of TieA by DNA-PK kinase, lane d represents DNA-PK (substrate) + TieA (enzyme) + ATP and indicated that TieA did not phosphorylate the DNA-PK substrate, lanes e and f represent negative controls. Y axis represents the relative light units (RLU). Data represent the mean ± SD of three independent experiments; two-tailed Student's *t* test was performed wherein $*P \leq 0.05$, $**P \leq 0.01$.

wards *tie*A during *H. pylori* infection was evaluated using RT^2 profiler array for a set of 84 genes majorly involved in innate immune responses directed against bacterial pathogens. Based on gene selection criteria ($P < 0.05$ and fold change ≥ 2), out of 84 genes tested, 30 genes majorly entailing pro-inflammatory cytokines such as IL-8, IL-6, CCL3, CCL5 and chemokines CXCL1, CXCL2 were significantly downregulated in AGS cells infected with *tie*A KO *H. pylori* (Figure 5) followed by some of the apoptotic genes (CARD6, CASP8 and JUN). Additionally, other genes related to pathogen recognition and triggering of innate responses were also observed to be downregulated such as MAP kinases (MAP2K1, MAP2K3, MAP2K4, MAP3K7, MAPK3 and MAPK8) (Figure 5). These results indicate that NF-κB (NF-κB-1, NF-κBia, Ikbkb) and MAPK are the key molecules involved in innate and antibacterial responses led by *tie*A. In line with above observations, we investigated the importance and role of TieA in *H. pylori* induced inflammatory responses at protein level in the infection process. To this end, we tested the isogenic *tie*A KO *H. pylori* for its ability to induce inflammatory response in AGS and THP-1 cell lines. Consistent with our previous findings, *tie*A KO exhibited significantly reduced ability to induce IL-8, IL-1β and IL-6 secretion in culture supernatants as compared to the WT *H. pylori* (Figure 6A). The RT^2 profiling and ELISA data complemented our results and demonstrated that TieA indeed stimulates the secretion of pro-inflammatory cytokines during *H. pylori* infection.

TieA mediated pro-inflammatory cytokine production is regulated *via* activation of AP-1

Many effector proteins of *H. pylori* are known to be involved in the activation of NF-κB and AP-1 (9–11,16,17) which further regulate the production of IL-8 and TNF-α (37). In congruence with this, a significant difference in NF-κB activation in AGS cells at mRNA level was observed when compared to WT and *tie*A KO *H. pylori* (Figure 5). However, when examined at protein level, nuclear translocation of p65 in AGS SIB02 cells (38) at 30 and 60 min post infection resulted in *tie*A KO *H. pylori* inducing the recruitment of p65 into the nucleus in a similar manner as that of WT *H. pylori* (Figure 6B). To obtain further evidence for NF-κB activation, we infected the AGS cells with WT and *tie*A KO *H. pylori* for 30 min followed by western blot analysis using phospho-p65 monoclonal antibody. WT and *tie*A KO *H. pylori* stimulated similar phosphorylation levels of p65 (Figure 6C). Additionally, we also investigated whether TieA could activate the AP-1 upon *H. pylori* infection. To address this, AGS cells were infected with both WT and *tie*A KO *H. pylori* for 45 and 60 min. Cell lysates were prepared and examined for the phosphorylation of c-Jun by ELISA and immunoblot analysis. No significant difference was observed in phosphorylation levels of both WT and TieA KO *H. pylori* infected AGS cells at 45 min post infection (Figure 6D and F). Nevertheless, this did not appear to be the case 60 min post infection, wherein *tie*A KO *H. pylori* produced significantly less phosphorylation of c-Jun as compared to the WT *H. pylori.* (Figure 6D and F). To further confirm the involvement of TieA in c-Jun activation, we treated the AGS cells with recombinant TieA (5 μg) protein for 60 min and probed against c-Jun by immunoblotting. It was observed that recombinant TieA induced phosphorylation of c-Jun (Figure 6E). These results suggest that TieA is required for AP-1 activation by *H. pylori*.

TieA induces cell death *via* Fas mediated apoptosis in gastric epithelial cells

Taking into account that TieA localizes to cytoplasm and nucleus of AGS cells, as described previously by us (17), cleaves naked and chromosomal DNA and phosphorylated by DNA-PK kinase, we pondered if TieA is capable of inducing apoptosis in gastric epithelial cells during *H. pylori* infection. To address this, AGS cells were infected with WT and *tie*A KO *H. pylori* for 24 h and double staining was carried out using annexin V-FITC and PI followed by flow cytometry. Figure 7A shows that, when AGS cells were infected with *tie*A KO *H. pylori*, 30.2% cells were stained positive for phosphatidylserine, a marker for early stages of apoptosis (right bottom quadrant), whereas in WT *H. pylori,* 41.8% cells were stained positive. These results demonstrated that AGS cells infected with *tie*A KO *H. pylori* led to a substantial decrease in annexin V-FITC binding. To further confirm the involvement of TieA in apoptotic cell death, we tested the ability of purified TieA protein (5 μg) to induce apoptosis. To ensure LPS free protein fraction, we treated the TieA protein with polymixin-B before application to gastric epithelial cells. Our results revealed that TieA significantly induced apoptosis in AGS cells (13%) as compared to the untreated AGS cells (Figure 7A and B). Moreover, heat inactivation of TieA abrogated the apoptosis inducing ability of TieA suggesting this property to be protein specific (Figure 7B). AGS cells treated with Staurosporine (50 nM) for 3 h were used as positive control. To obtain further evidence for cell death induction, we analyzed the ability of TieA to induce histone release. A significant reduction in the histone release in AGS cells infected with *tie*A KO was observed as compared to the WT *H. pylori* (Figure 7C). However, the apoptosis inducing ability of *tie*A KO *H. pylori* was not completely abolished, possibly due to the presence of other virulence factors such as *vac*A, urease and γ-glutamyl transpeptidase which also contribute to cell death. Additionally, we also looked for the expression of Fas receptors on AGS cells following treatment with purified TieA (5 μg). Expression of Fas receptors on TieA treated AGS cells was significantly higher than unstimulated cells (Figure 7D). These results conveyed that upregulation of cell death surface receptor Fas by TieA increases the susceptibility of AGS cells to undergo Fas dependent apoptotic cell death.

TieA induces mitogen-activated protein kinase (MAPK) activation

JNK and p38 MAPK are known to be activated in response to diverse cellular and environmental stresses such as DNA damage, oxidative stress and pro-inflammatory cytokine functions (39,40). Thus, we examined the activation of ERK, p38 and JNK in response to TieA in infected AGS cells. AGS cells were co-cultured with WT and *tie*A KO

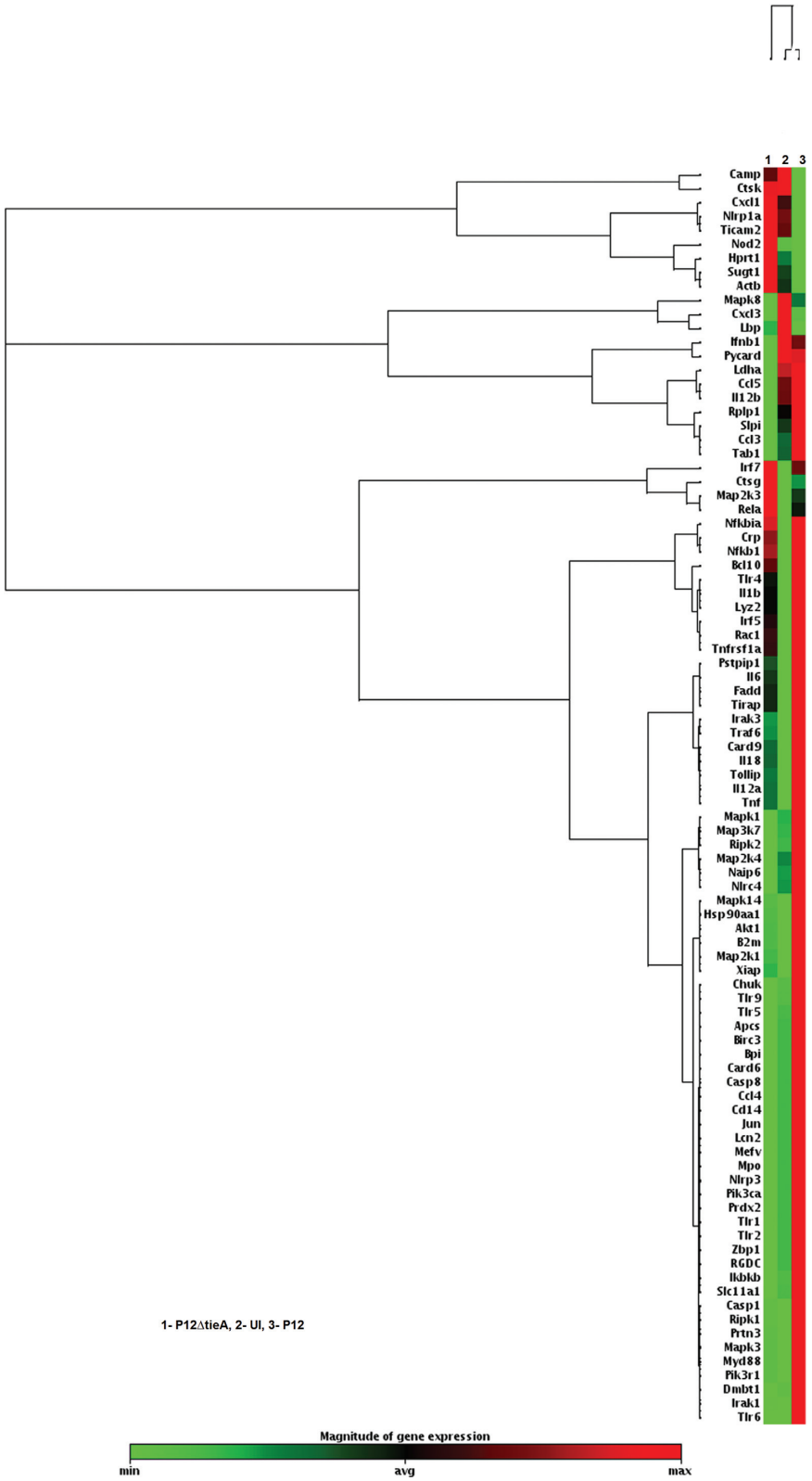

Figure 5. RT^2 profiler antibacterial gene expression analysis. AGS cells were infected with P12 and *tie*A KO *H. pylori* for 3 h. Total RNA was extracted, reverse transcribed, and subjected to RT^2 PCR array. The quantification of induced genes was determined *via* RT^2 profiler PCR array data analysis software V4 (Qiagen). Magnitude of red color represents higher levels of gene expression; green color represents low gene expression levels. Details of the genes analyzed are provided in Supplementary Table S3. UI: uninfected.

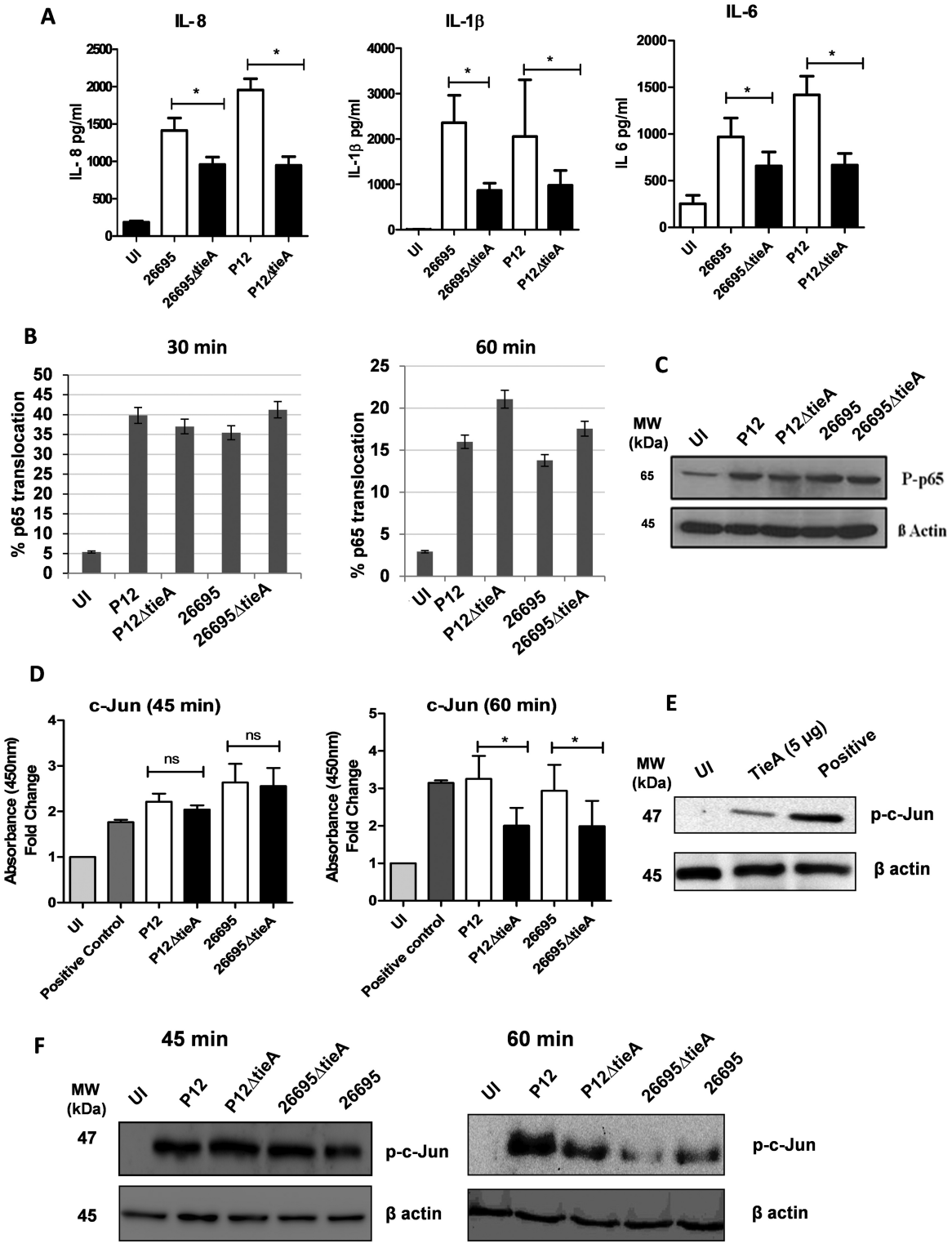

Figure 6. (**A**) Estimation of IL-8 (AGS cells), IL-1β and IL-6 (THP-1 cells) levels in culture supernatants post infection with WT and *tie*A KO *H. pylori* as indicated (by ELISA). (**B**) NF-κB activation in AGS SIB02 cells. AGS cells were infected for 30 min and 60 min and percentages of p65 translocation were measured. (**C**) Immunoblot analysis of phospho-p65 and β actin protein levels in both uninfected and infected AGS cells (infected for 30 min). *H. pylori* infected AGS cells were lysed in 2X Laemmli buffer and boiled for 10 min at 95°C, followed by immunoblotting. (**D**) TieA signals *via* activation of c-Jun. AGS cells were infected (for 45 min and 60 min) and cell lysate was prepared as described in materials and methods section. Cell lysate was tested for c-Jun activation by ELISA as fold change (fold change = average of test OD/average of uninfected sample's OD). Positive control was kit specific and prepared from various cell types (**E**) c-Jun phosphorylation by TieA in AGS cells. (**F**) Immunoblot analysis for c-Jun activation. Cell lysate was prepared and loaded on 12% SDS-PAGE and probed with monoclonal c-Jun antibody. Data represent the mean ± SD of three independent experiments; two-tailed Student's *t* test was performed wherein $*P \leq 0.05$, $**P \leq 0.01$. UI: uninfected, kDa: kilodalton and ns: non-significant.

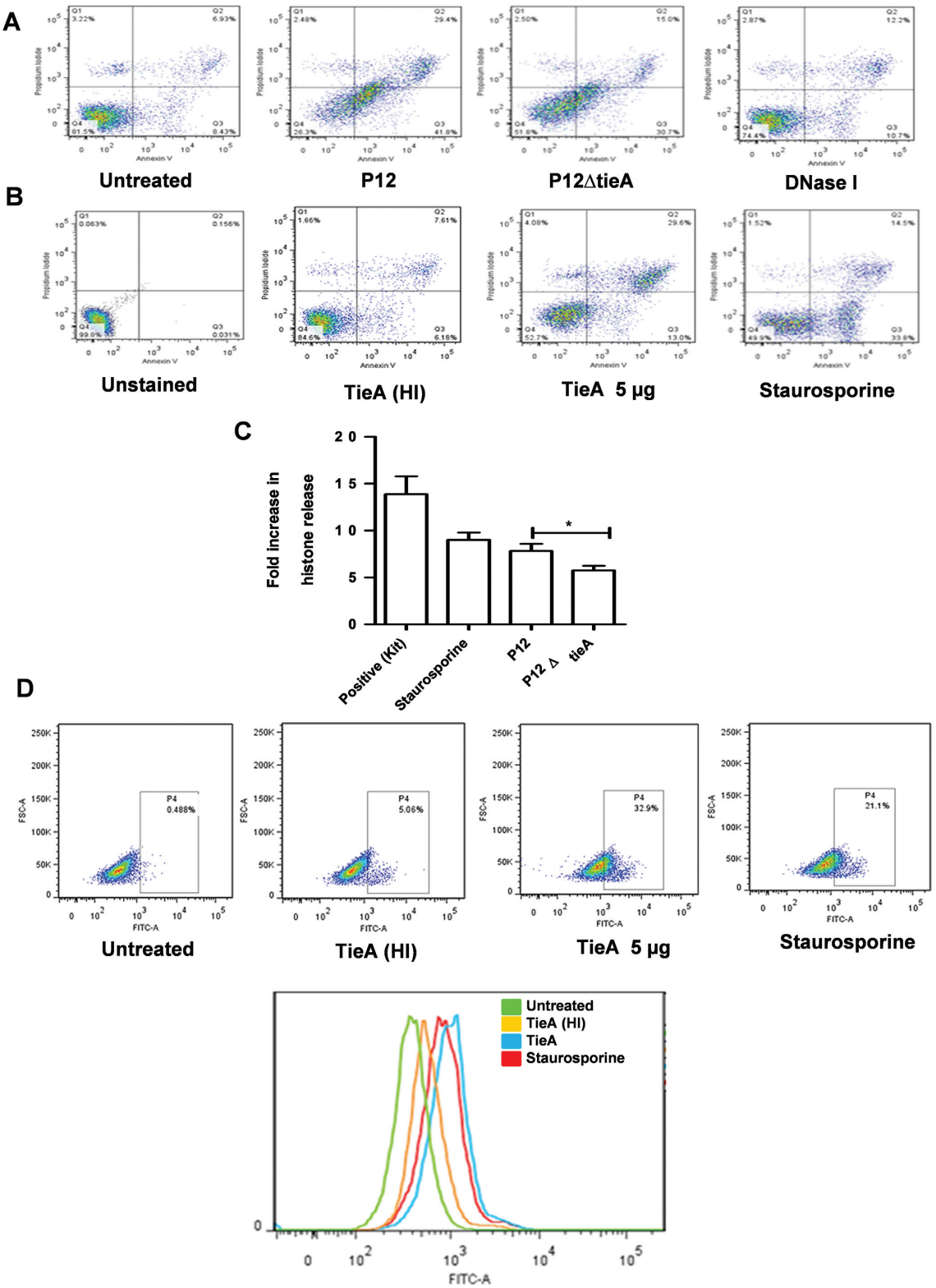

Figure 7. (**A**) Measurement of cell death by Annexin V-FITC/PI staining. AGS cells were infected (WT and KO strains of *H. pylori*) or treated (5 μg of TieA protein) for 24 h as indicated and were examined for apoptotic cells using Annexin V-FITC apoptosis detection kit as described in materials and methods section. Staurosporine and DNase I treated AGS cells were used as positive controls. (**B**) Annexin V-FITC/PI staining to analyze apoptosis in AGS cell line induced by TieA using flow cytometry. (**C**) AGS cells were infected with *H. pylori* (WT or KO) at an MOI of 100 for 24 h, and cell death was measured as fold change in histone release. Data are mean ± SD of three independent experiments; two-tailed Student's *t* test was performed for statistical analysis, $*P \leq 0.05$. (**D**) Flow cytometry analysis showing expression of Fas receptors on AGS cells upon treatment with TieA (5 μg) after 24 h. The shift in the histogram peak for TieA and staurosporine as compared to the untreated AGS cells indicates an enhanced expression of Fas receptors on AGS cells. HI: heat inactivated.

H. pylori for 45 min and the cell lysates were tested for phospho-ERK, phospho-p38 and phospho-JNK by ELISA and immunoblotting. Cells infected with WT *H. pylori* resulted in elevated levels of phospho-p38 and phospho-JNK as compared to the AGS cells infected with *tie*A KO *H. pylori* (Figure 8A, B). In contrast, there was no significant difference in the activation levels of phospho-ERK between cells infected with WT *H. pylori* and *tie*A KO thereby confirming that TieA selectively activates p38 and JNK without affecting ERK (Figure 8A and B). To further assess the role of TieA in activation of p38 and JNK, AGS cells were treated with specific inhibitors of either p38 or JNK before treatment with TieA (5 μg) protein. When the AGS cells were pretreated for 1 h with 10 μM p38 inhibitor (SB203580) and 30 μM JNK inhibitor (SP600125), phosphorylation levels of p38 and JNK were comparable to that of untreated cells (Figure 8C). The phospho-p38 and phospho-JNK levels produced in cells pre-incubated with p38 and JNK inhibitors followed by stimulation with TieA (5 μg) were significantly lower as compared to the TieA treated cells alone (Figure 8C). These results illustrate that TieA is involved in p38 and JNK activation by *H. pylori*.

DISCUSSION

The spectrum of disease manifestations caused by *H. pylori* is attributable to various secretory effector proteins (9–11,28,41). Despite triggering vigorous innate and adaptive immune responses, these secreted effector proteins also facilitate survival of the pathogen in the gastric mucosa (9,16). Bacterial species produce multi-functional proteins capable of delivering diverse effects on different cell types and tissues. It has become apparent that many bacterial moonlighting proteins potentially function across bacterial and host interface by modulating mammalian cellular responses in order to ensure colonization and persistence of the pathogen inside host. Furthermore, genes encoding multi-functional proteins have additional benefits such as they obviate the need for bacterial genome expansion and accommodate several multipronged functions, in less genomic space. DNA dependent phosphorylation of proteins is known for several years now (42) and the same has enormous regulatory significance with respect to cellular homeostasis in healthy as well as infected tissues. In the current study, we attempted to decipher the molecular and cellular mechanisms that underline the multipronged actions of a bacterial endonuclease (TieA) involved in the pathogenesis of *H. pylori*. *tie*A is a strain specific gene, which is present among 33% of all the sequenced *H. pylori* genomes. Our analysis revealed that it was mostly prevalent in strains belonging to hpEurope, hpSouthIndia and hspAmerind lineages but was completely absent in the East Asian strains (Figure 1A). These findings are in line with our previous reports, wherein prevalence of *tie*A was found to be 32% in *H. pylori* isolates obtained from multiethnic Malaysian population and was highest among the Indian ethnic group (non-East Asian lineage) (17). Recently, Kumar *et al*. have also demonstrated that out of 27 Malaysian strains, *tie*A was present in 11 strains which specifically belonged to non-east Asian genotype (43). These observations further validate the diversity among different strains of *H. pylori* concerning the presence/absence of strain specific genes.

Many bacterial pathogens identify environmental factors as signals that are characteristic of the host and accordingly fine–tune their transcriptome (44,45). One of the pivotal environmental determinants associated with an enhanced gastric cancer risk is the intake of high levels of dietary salt (46). We also observed that the transcription of *tie*A was strongly upregulated in response to high salt (NaCl) concentration and low pH (Figure 1B and C). Similarly, several such genes as *hp0010*, *hp1024*, *hp1021*, different heat shock proteins, virulence genes such as *cag*A, *vac*A and outer membrane protein genes have been previously reported to be positively regulated in response to high salt concentration and low pH (47,48). A recent study by Loh *et al.* demonstrated that variation in TAATGA DNA motif was responsible for salt induced upregulated expression of *cag*A in *H. pylori* (48). However, the exact molecular mechanism by which DNA motifs regulate *cag*A expression in response to salt remains unclear. In addition to the environmental factors, contact with host cells also modulates gene expression profiles in several bacterial species including *H. pylori* (49). Likewise, we identified that the expression of *tie*A was strongly induced (Figure 1D) in *H. pylori* akin to *ice*A, *cag*A *and vac*A post adherence to AGS cells. (31). The differential expression of *tie*A in response to high NaCl, low pH and host cell contact might be a mechanism for the regulation of virulence and stress survival; this may also confer fitness benefits to *H. pylori* populations for adaptation to frequent environmental changes. However, mechanisms underlying the regulation of altered gene expression remain unidentified. Whole genome analysis of *H. pylori* revealed the presence of 10 R-M genes that exhibit phase variation. These genes contain homopolymeric tracts or dinucleotide repeats of length varying from 5–15 base pairs and have comparatively higher mutation rates than other part of the genome (7,50). Slipped strand mispairing in these repeats is reported as one of the mechanisms of genotypic and phenotypic variation (51). Similarly, a plausible mechanism for differential expression of *tie*A could be the presence of poly A (A8) tract of eight residues (Supplementary Figure S4) within the coding sequence of *tie*A which probably leads to variation in *tie*A gene expression.

Intriguingly, Interpro analysis of TieA protein sequence indicated presence of a domain of unknown function, DUF1887, in addition to restriction endonuclease and t-RNA endonuclease domains. The DUF is widely reported in hypothetical bacterial proteins and in particular DUF1887 was reported to be present in R-M genes along with a CRISPR associated (Cas) gene (http://pfam.xfam.org). Therefore, it can be surmised that TieA could be involved in DNA binding and possesses an endonuclease activity. Accordingly, our results have elucidated the binding and cleavage activity of TieA with linear and circular DNA molecules, irrespective of sequence specificity, similar to the activity of DNase I (Figure 3A–D). Most of the apoptotic DNases such as DFF40 and EndoG exhibit divalent cation (Mg^{2+}) dependent endonucleases activity, however, DNase I requires both Ca^{2+} and Mg^{2+} for DNA hydrolysis (52,53). Our findings demonstrated similar co-factor requirements of TieA (Ca^{2+}–Mg^{2+}-dependent endonuclease

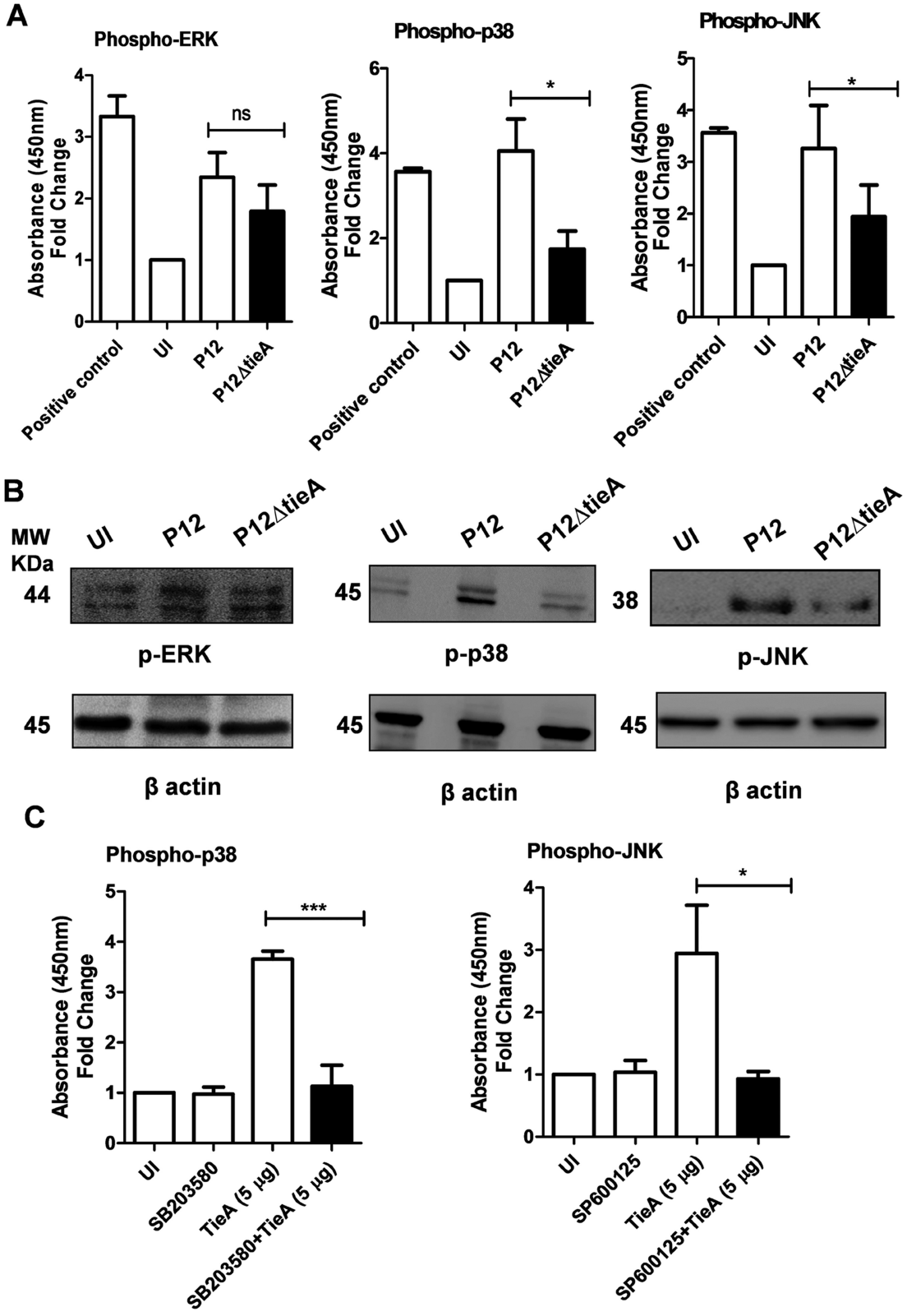

Figure 8. (**A**) MAP kinase activation: AGS cells were infected with P12 and *tie*A KO *H. pylori* at an MOI of 100 for 45 min and cell lysate was prepared. Cell lysate was tested for the activated phospho-ERK, phospho-p38 and phospho-JNK by ELISA. The values are expressed as fold change. (**B**) Immunoblot analysis of phospho-ERK, phospho-p38 and phospho-JNK protein levels in infected AGS cells. (**C**) AGS cells were left untreated or were pretreated with p38 inhibitor SB203580 (10 μM) and JNK inhibitor SP600125 (30 μM) for 1 h, prior to treatment with TieA for 2 h in presence of inhibitors. Levels of phosphorylated p38 and JNK in the cell lysates were quantitated as fold change (values are expressed as the fold increase relative to the level in untreated cells). Data are shown as mean ± SD of three independent experiments; two-tailed Student's *t* test was performed, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, ns: non-significant, UI: uninfected.

activity). In addition to this, TieA degrades chromosomal DNA in prefixed AGS cells (Figure 4A). Many of the endonucleases are reported to be secreted extracellularly in culture supernatant (20,22,23). We also identified extracellular secretion of TieA in broth supernatant of *H. pylori* culture (Figure 4B) and detected its translocation into the AGS cells (Figure 4C). The underlying secretion mechanisms of many *H. pylori* proteins, including TieA are currently unclear. However, from our findings, it was evident that the translocation of TieA into gastric epithelial cells was independent of T4SS (Figure 4C). Because TieA is devoid of a signal peptide, its mechanism of secretion may involve either a sec independent secretion system or some other unknown pathways. Similarly, proteins such as HP0902, thioredoxin, single stranded-DNA-binding protein, histone like DNA-binding protein and ribosomal protein L11 were secreted into the supernatant of *H. pylori* despite lacking a signal peptide (54). However, irrespective of known secretion mechanisms, extracellular proteins of *H. pylori* could mediate host-pathogen interaction when they come in close vicinity of host cellular compartments. This raises the question as to what role do these 'extracellular endonucleases' play in *H. pylori* pathogenesis?

The role of TieA in pathogenesis of *H. pylori* could possibly be explained by the endonuclease activity of TieA, which cleaves DNA nonspecifically and would aid in degradation or solubilization of mucus by acting on the digestion of sloughed cells as well as in the degradation of extracellular host cell DNA. The endonuclease function also points to the role of TieA in natural transformation as similar findings were observed by O'Rourke *et al*, wherein, an open reading frame (ORF) *hp0323* from *H. pylori* that encodes a nuclease protein was shown to be involved in natural transformation (55). Moreover, future studies involving confirmation of the role of TieA in transformation are pertinent. *H. pylori* has also been shown to utilize NucT nucleases to retrieve purines from extracellular host cell DNA to cater its growth requirements (56).

Bacterial nucleases assist in natural transformation; however, eukaryotic DNases/nucleases have been reported as important players in inducing apoptosis in various cell types (19). Taking into account the DNA cleavage function and the ability of TieA to undergo nucleo-cytoplasmic shuttling in gastric epithelial cells (17), we can possibly envision its role in maintenance or prolongation of host pathophysiology by apoptosis of the host tissues. Infection experiments in AGS cells with WT and *tie*A KO *H. pylori* showed an important role of TieA in induction of apoptosis during *H. pylori* infection. The significant reduction in histone release and annexin binding to AGS cells by *tie*A KO *H. pylori* confirmed apoptosis inducing ability of TieA (Figure 7A, B and C). Moreover, AGS cells treated with recombinant TieA underwent apoptosis through Fas activation, suggesting that TieA induces Fas mediated apoptosis in AGS cells which was specific to TieA protein (Figure 7D). This is in agreement with our previous study where TieA was shown to induce Fas mediated apoptosis in THP-1 cells *via* TNFR-1 interaction (16). Although TieA is devoid of nuclear localization signal (NLS), studies of Butt *et al.* suggest that IGFBP-3 promotes apoptosis independent of a nuclear localization signal (57). Therefore, it is possible that TieA might trigger apoptosis without being internalized and by following some unconventional pathway(s) that promotes cell death in a cell specific manner. These results reinforce that TieA might also promote apoptosis of host cells *via* its endonuclease function and thus serves as apoptotic deoxyribonulease.

We also showed that TieA undergoes post translational modifications, such as phosphorylation, and contains consensus phosphorylation sites for several protein kinases. Protein phosphorylation and dephosphorylation are key contributors in regulating the activity of various transcription factors in response to environmental stimuli (58). We demonstrated that TieA is capable of autophosphorylation as a serine protein kinase and also gets phosphorylated by DNA- dependent protein kinase (DNA-PK) as demonstrated when tested through *in vitro* kinase assay (Figure 4D-E). DNA-PK is a nuclear enzyme which requires DNA for its kinase activity (59). The kinase activity of DNA-PK is required for DNA double-strand break (DSBs) repair and eliciting apoptosis in response to acute DNA damage (59). Although DNA-PK is predominantly a nuclear kinase, it was also found in lower amounts in the cytoplasm of hepatoma and HeLa cells (42,60). These findings complement our observations that TieA gets translocated into the AGS cells (Figure 4C) and localizes in cytoplasm and nucleus of the AGS cells (18) wherein it may get phosphorylated by DNA-PK kinase and may act as an effector for inducing apoptosis of the host cells. We further attempted to delineate the signaling pathway(s) involved in TieA mediated pathogenesis. Generally, mitogen activating protein kinase (MAPK) pathway is associated with apoptosis and inflammation progression, which mainly consists of ERK, p38 and JNK (40). Distinct MAPK pathways determine the cell fate, either by playing a protective role or otherwise, depending upon the type of stimuli (40). ERKs are involved in cell survival, whereas JNK and p38 mainly entail stress responses and are involved in apoptosis (61). We have demonstrated in this study that infection with isogenic KOs of *tie*A impairs p38 and JNK mediated signaling but had no effect on ERK signaling (Figure 8). It has been shown that p38 and JNK induce secretion of pro-inflammatory cytokines while ERK induces anti-inflammatory cytokine secretion in *Leishmania major* infections (62). Events such as activation of JNK and p38-MAPK pathways and simultaneous inhibition of ERK pathway are cardinal phenomena for the initiation of apoptosis (40). Our results demonstrated that JNK activation was accompanied by activation of c-Jun, a component of AP-1, and acts as a pro-apoptotic effector (63). This has also been described previously with CagA and VacA effector proteins of *H. pylori.* However, these proteins activate ERK pathway as well (64). In line with our previous findings, we found that TieA induced NF-κB activation in AGS cells; however, TieA isogenic mutants also induced NF-κB activation similar to the TieA WT strains (Figure 6C), probably due to the presence of other virulence proteins involved in NF-κB induction. Also, it appears that the inflammatory responses activated by TieA are dually regulated by NF-κB and AP-1 to facilitate overall transcription efficiency. Therefore, it can be noticed that multiple signaling events are activated during *H. pylori* infections that are collectively required to initiate a successful chronic inflammation throughout the infection period.

Additionally, *tie*A influences expression of major virulence factors such as *cag*A and *vac*A. *tie*A deletion upregulates *cag*A and *vac*A mRNA expression, though *tie*A does not perturb formation of humming bird phenotype in AGS cells (Figure 2A–D). Moreover, knockout of *cag*E gene which is a component of T4SS assembly did not cause any upregulation of *tie*A gene expression and vice versa. A more plausible explanation by which loss of *tie*A or *cag*A alters and compensates each other's expression would be that the additional genes entailing similar functions may compensate for the knockout gene, either by delaying consequences or by completely countervailing the deficiency by upregulating expression of genes which can rescue the loss of function in the genome (65). Because, TieA can function both as a pro-inflammatory and a pro-apoptotic protein, deletion of either *cag*A or *vac*A or both could lead to an upregulated expression of *tie*A to counterbalance the loss of *cag*A and/or *vac*A in the genome. However, these alternations did not correlate with observed differences at the level of humming bird formation. This discrepancy may be attributable to the fact that TieA does not play a direct role in induction of humming bird formation; due to this, even though the mRNA expression of *tie*A was upregulated in *cag*A knockout *H. pylori,* humming bird formation remained unaltered. Moreover, this hints towards the possibility that TieA acts as a seasoned virulence factor harnessed by the pathogen to complement the deficiencies of *cag*A and *vac*A at the level of inflammation and apoptosis. This notion is supported by studies which reported that pathology of *H. pylori* infection remains unaffected even in the absence of *cag*A and *vac*A, as reviewed elsewhere (66) or when these virulence genes undergo genetic rearrangements (67).

Taken together, our study envisages a role of TieA in induction of gastric inflammation and apoptosis. We demonstrated that TieA mediates gastric epithelial cell apoptosis by acting as an endonuclease (apoptotic deoxyribonuclease). The underlying mechanisms and presumable events might include (i) upregulated expression, (ii) translocation into the host cell, (iii) inflammatory cytokine secretion, (iv) phosphorylation and (v) binding to/digestion of DNA; these events are eventually likely involved in activating TieA to promote apoptosis. In future, it will be possible to gather further evidence to prove the above events collectively or individually in different animal models, although these multiple possibilities and the observed pleiotropic effects of TieA probably suggest that the latter appear to groom and fine tune *H. pylori* strains towards adaptation and long term persistence inside their host.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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