Helicobacter pylori CagA activates NF-κB by targeting TAK1 for TRAF6-mediated Lys 63 ubiquitination

Acacia Lamb[1], Xiao-Dong Yang[1], Ying-Hung N. Tsang[1], Jiang-Dong Li[2], Hideaki Higashi[3], Masanori Hatakeyama[3], Richard M. Peek[4], Steven R. Blanke[5] & Lin-Feng Chen[1+]

[1]Department of Biochemistry, College of Medicine, University of Illinois at Urbana-Champaign, Urbana, Illinois, USA, [2]Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, New York, USA, [3]Division of Molecular Oncology, Institute for Genetic Medicine and Graduate School of Science, Hokkaido University, Sapporo, Japan, [4]Department of Medicine, Division of Gastroenterology, Vanderbilt University School of Medicine, Nashville, Tennessee, USA, and [5]Department of Microbiology, University of Illinois at Urbana-Champaign, Urbana, Illinois, USA

Helicobacter pylori-initiated chronic gastritis is characterized by the *cag* pathogenicity island-dependent upregulation of proinflammatory cytokines, which is largely mediated by the transcription factor nuclear factor (NF)-κB. However, the *cag* pathogenicity island-encoded proteins and cellular signalling molecules that are involved in *H. pylori*-induced NF-κB activation and inflammatory response remain unclear. Here, we show that *H. pylori* virulence factor CagA and host protein transforming growth factor-β-activated kinase 1 (TAK1) are essential for *H. pylori*-induced activation of NF-κB. CagA physically associates with TAK1 and enhances its activity and TAK1-induced NF-κB activation through the tumour necrosis factor receptor-associated factor 6-mediated, Lys 63-linked ubiquitination of TAK1. These findings show that polyubiquitination of TAK1 regulates the activation of NF-κB, which in turn is used by *H. pylori* CagA for the *H. pylori*-induced inflammatory response.
Keywords: CagA; NF-κB; TAK1; TRAF6/ubiquitination

INTRODUCTION

Helicobacter pylori infection causes chronic gastritis and peptic ulceration, and is the strongest risk factor for the development of gastric cancer (Peek & Blaser, 2002; Hatakeyama, 2008).

The inflammatory response associated with gastritis is characterized by the expression of various proinflammatory cytokines, and transcription factor nuclear factor (NF)-κB has an important role in the regulation of their expression (Ghosh & Karin, 2002). *H. pylori* infection activates NF-κB and the expression of interleukin-8 (IL-8) in epithelial cells, which is believed to be critical for *H. pylori*-initiated chronic inflammation (Sharma *et al*, 1998; Maeda *et al*, 2001; Naumann, 2001; Hirata *et al*, 2006).

The *H. pylori cag* pathogenicity island is required for activation of NF-κB and expression of NF-κB target genes (Glocker *et al*, 1998; Foryst-Ludwig & Naumann, 2000), and its encoded virulence factor, CagA, has an important role in the pathogenicity of *H. pylori*, including *H. pylori*-induced inflammation (Brandt *et al*, 2005; Kim *et al*, 2006). Infection with *H. pylori cagA*-positive strains is associated with higher grades of gastric inflammation and an increased risk of gastric cancer than infection with *cagA*-negative strains (Blaser *et al*, 1995; Parsonnet *et al*, 1997), thereby highlighting the role of CagA in the *H. pylori*-mediated inflammatory response. However, the exact function of CagA in the activation of NF-κB and the NF-κB-dependent inflammatory response is yet to be addressed.

CagA is injected into *H. pylori*-infected host epithelial cells—by using a type-IV secretion system—where it is often activated by tyrosine phosphorylation through the host src kinase and targets host proteins to modify cellular responses (Peek, 2005; Hatakeyama, 2008). For example, phosphorylated and non-phosphorylated CagA interact, respectively, with Src homology 2 domain-containing protein tyrosine phosphatase (SHP2) and with host proteins such as growth-factor-receptor-bound protein 2 (GRB2) and zona occludens 1 (ZO-1) to induce abnormal proliferation and movement of gastric epithelial cells (Mimuro *et al*, 2002; Amieva *et al*, 2003; Hatakeyama, 2004). Non-phosphorylated CagA also destabilizes the E-cadherin/β-catenin complex to induce the activation of the β-catenin signal that underlies intestinal metaplasia (Franco *et al*, 2005; Murata-Kamiya *et al*, 2007). However, the cellular

[1]Department of Biochemistry, College of Medicine, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801 USA
[2]Department of Microbiology and Immunology, University of Rochester Medical Center, New York 14642, USA
[3]Division of Molecular Oncology, Institute for Genetic Medicine and Graduate School of Science, Hokkaido University, Sapporo 060-0815, Japan
[4]Department of Medicine, Division of Gastroenterology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232, USA
[5]Department of Microbiology, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801, USA
[+]Corresponding author. Tel: +1 217 333 7764, Fax: +1 217 244 5858;
E-mail: lfchen@life.uiuc.edu
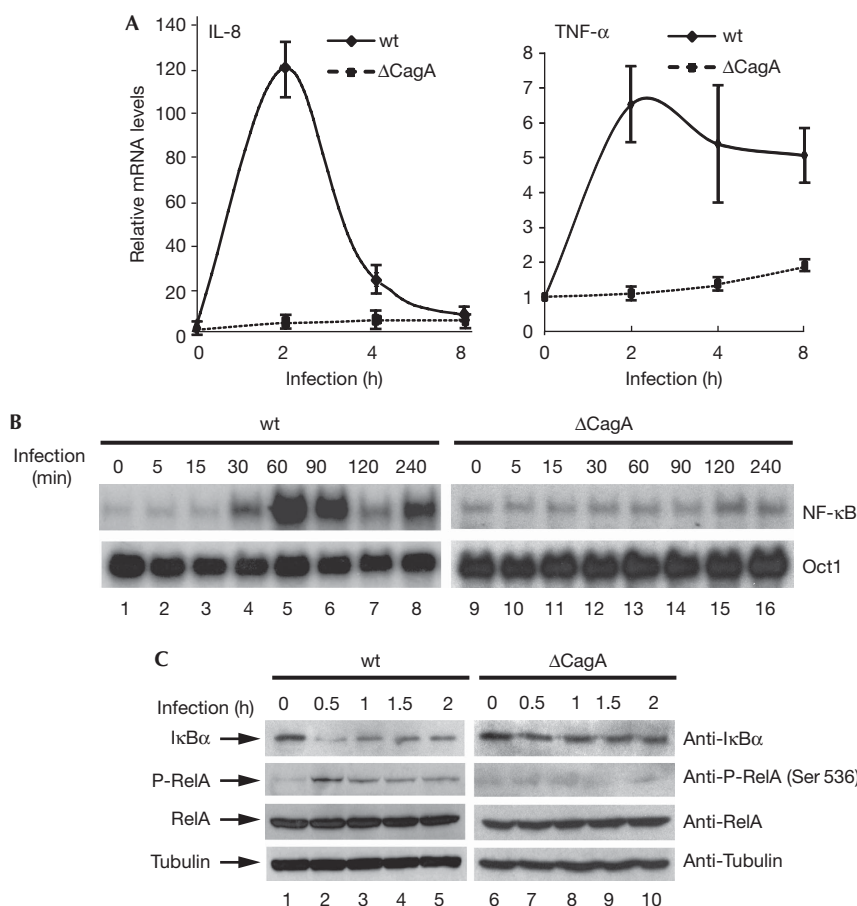
Fig 1 | CagA is required for *Helicobacter pylori*-induced activation of NF-κB. (**A**) AGS cells were infected with G27 wt or ΔCagA *H. pylori* and quantitative RT–PCR was performed to analyse the expression of NF-κB target genes IL-8 and TNF-α. (**B**) EMSA was performed using whole-cell extracts from AGS cells infected with wt or ΔCagA *H. pylori* by using radiolabelled NF-κB and Oct1 probes. (**C**) Levels of IκBα, phosphorylated Ser 536 RelA, RelA, CagA and tubulin were detected in lysates from AGS cells infected with G27 wt or ΔCagA *H. pylori* by immunoblotting with the indicated antibodies. EMSA, electrophoretic mobility-shift assay; IL-8, interleukin-8; NF-κB, nuclear factor-κB; RT–PCR, real-time PCR; TNF-α, tumour necrosis factor-α; wt, wild type.

proteins CagA targets that are involved in inducing the inflammatory response remain elusive.

NF-κB has an important role in the regulation of inflammatory responses in mammals. The prototypical NF-κB complex, which is a heterodimer of p50 and RelA, is sequestered in the cytoplasm by its inhibitor IκBα. On stimulation, the IκB kinase (IKK) complex is activated, leading to phosphorylation and degradation of IκBα, nuclear translocation of NF-κB and activation of its target genes (Ghosh & Karin, 2002). Transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1) is a key regulator of signal transduction cascades that lead to stimulus-coupled phosphorylation and activation of IKK (Adhikari *et al*, 2007). Emerging evidence indicates that activation of TAK1 and IKK is regulated by tumour necrosis factor receptor-associated factor 6 (TRAF6)-mediated, Lys 63-linked ubiquitination of TRAF6, interleukin 1 receptor-associated kinase 1 and NF-κB essential modulator (Adhikari *et al*, 2007). Recent studies also show that TAK1 is polyubiquitinated by TRAF6 in response to TGF-β, and that Lys 63-linked ubiquitination is required for TGF-β-induced activation of p38/Jun N-terminal kinase and AP-1 (Sorrentino *et al*,

2008), indicating that TAK1 ubiquitination might also be crucial for the activation of IKK and NF-κB.

Here, we show that CagA is essential for *H. pylori*-induced activation of NF-κB, and identify TAK1 as a new cellular target of CagA.

RESULTS AND DISCUSSION
H. pylori-mediated activation of NF-κB requires CagA
To investigate the role of CagA in the *H. pylori*-induced inflammatory response, we compared wild-type (wt) and *cagA*-deficient *H. pylori* for their ability to induce the expression of inflammatory genes in AGS gastric epithelial cells. Infection of AGS cells with wt *H. pylori* strain G27, but not its *cagA*-deficient isogenic mutant, induced the expression of IL-8 and tumour necrosis factor-α (TNF-α) messenger RNA (mRNA; Fig 1A). Similar results were obtained with two other *H. pylori* strains, NCTC11637 and 7.13 (supplementary Fig S1 online). These data indicate that induction of proinflammatory genes can be generalized for most *cagA*-positive *H. pylori* strains and that the defect from the *cagA*-deficient mutants is unlikely to be due to a polar effect.
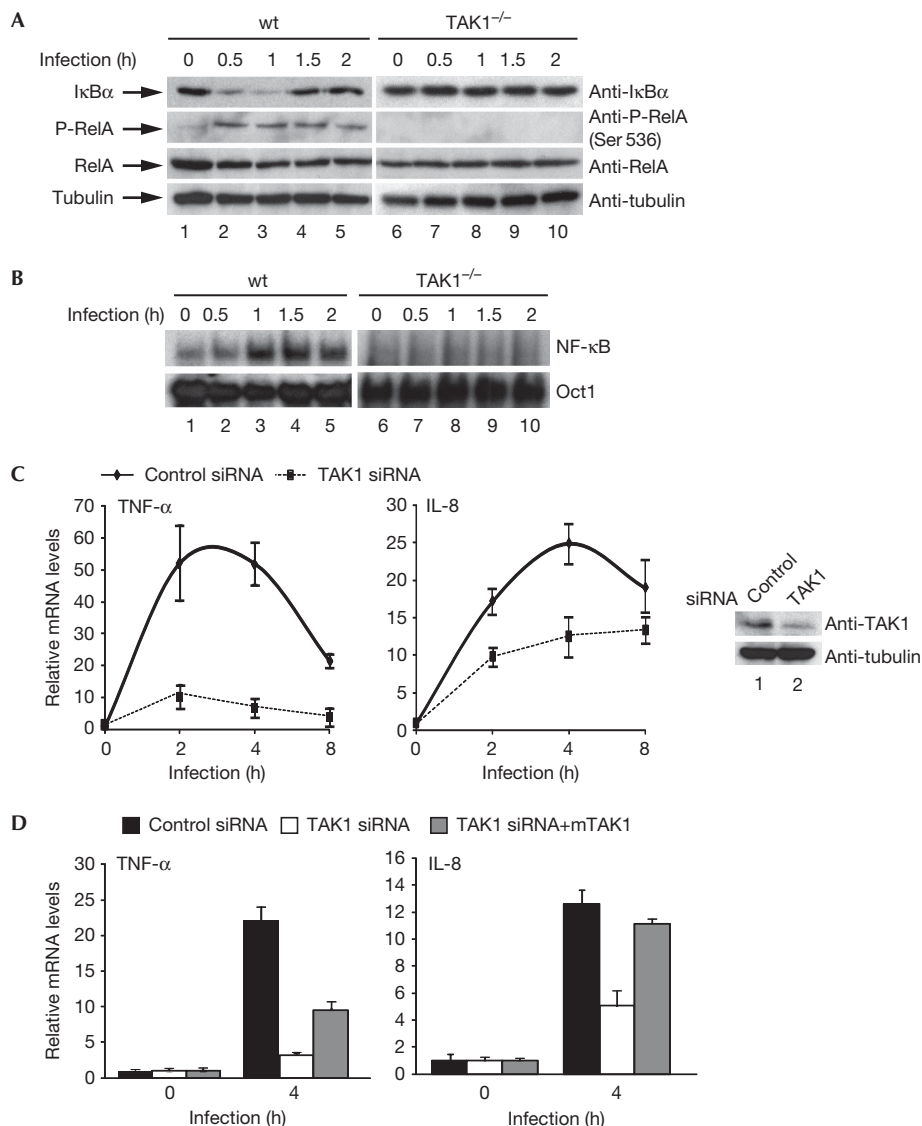
Fig 2 | TAK1 is required for *Helicobacter pylori*-induced NF-κB activation. (A) Levels of IκBα, phosphorylated Ser 536 RelA, RelA, TAK1 and tubulin were detected in the lysates from wt or TAK1$^{-/-}$ MEFs infected with *H. pylori*. **(B)** EMSA was performed using whole-cell extracts from wt or TAK1$^{-/-}$ MEFs infected with *H. pylori*, as in Fig 1B. **(C)** AGS cells transfected with control or TAK1 siRNA were infected with *H. pylori* and RT–PCR was performed to analyse NF-κB target gene expression. Levels of TAK1 and tubulin are shown in the right panels. **(D)** AGS cells transfected with control siRNA or TAK1 siRNA were reconstituted with mouse TAK1 (mTAK1), followed by infection for 4 h with *H. pylori*. Gene expression was analysed as shown in **(C)**. EMSA, electrophoretic mobility-shift assay; IL-8, interleukin-8; MEF, mouse embryonic fibroblast; NF-κB, nuclear factor-κB; RT–PCR, real-time PCR; siRNA, short interfering RNA; TAK1, transforming growth factor-β-activated kinase 1; TNF-α, tumour necrosis factor-α; wt, wild type.

As NF-κB regulates the expression of many inflammatory cytokines, including IL-8 and TNF-α, we next investigated whether *H. pylori* infection activated NF-κB and whether this activation was CagA-dependent. wt *H. pylori*, but not the *cagA*-deficient mutant, activated all three κB luciferase reporters, including two that contain natural promoters from NF-κB target genes IL-8 and E-selectin (supplementary Fig S2 online). Furthermore, only wt *H. pylori*, but not the *cagA*-deficient mutant, stimulated the binding of NF-κB, heterodimers of RelA and p50, to DNA (Fig 1B; supplementary Fig S3 online).

To define the signalling pathway leading to the activation of NF-κB, we examined the degradation and resynthesis of IκBα and phosphorylation of RelA in AGS cells infected with wt and *cagA*-deficient *H. pylori*. IκBα was degraded and resynthesized in cells infected with wt but not in those infected with *cagA*-deficient *H. pylori* (Fig 1C). In addition, wt *H. pylori*, but not the *cagA*-deficient mutant, stimulated the phosphorylation of RelA at Ser 536, which is also mediated by activated IKK (Fig 1C). Altogether, these data indicate that *H. pylori* infection stimulates CagA-dependent activation of NF-κB through the activation

of IKK. Other studies also indicate that CagA induces cytokine release through activation of NF-κB (Brandt *et al*, 2005; Shibata *et al*, 2006); although some reports indicate that CagA might not be required for activation of NF-κB (Viala *et al*, 2004), this discrepancy is due to the different cell lines used in the studies. In 293 cells and macrophages, CagA does not seem to be essential for activation of NF-κB (Maeda *et al*, 2001; Viala *et al*, 2004). However, in gastric epithelial cells—the most physiologically relevant cells—CagA is essential for activation of NF-κB (Fig 1; Brandt *et al*, 2005).

## TAK1 is required for NF-κB activation by *H. pylori*

As TAK1 is the upstream kinase for IKK (Wang *et al*, 2001)—and is also used by effectors from other pathogens, such as human T-lymphotrophic virus-1 protein TAX (Wu & Sun, 2007)—we next evaluated the role of TAK1 in the *H. pylori*-induced activation of IKK and NF-κB. When wt or TAK1-deficient (TAK1$^{-/-}$) mouse embryonic fibroblasts (MEFs) were infected with *H. pylori*, IκBα was degraded and resynthesized, and phosphorylation of Ser 536 of RelA was induced in wt MEFs but not in TAK1$^{-/-}$ MEFs (Fig 2A). Furthermore, *H. pylori* stimulated the DNA-binding activity of NF-κB in wt but not in TAK1$^{-/-}$ MEFs (Fig 2B). These data indicate that TAK1 is essential for the activation of IKK and NF-κB after *H. pylori* infection. Consistently, infection of wt MEFs, but not TAK1$^{-/-}$ MEFs, with *H. pylori* stimulated mRNA expression of TNF-α, IL-6 and E-selectin—three NF-κB target genes (supplementary Fig S4 online).

To demonstrate further the role of TAK1 in *H. pylori*-induced NF-κB activation, we knocked down the expression of TAK1 in AGS cells. Depletion of TAK1 by short interfering RNA (siRNA) impaired the phosphorylation of RelA (supplementary Fig S5 online) and *H. pylori*-induced mRNA expression of TNF-α and IL-8 (Fig 2C). The partial decrease in expression of TNF-α and IL-8 mRNA in TAK1-knockdown cells (compared with TAK1$^{-/-}$ MEFs) probably reflects the incomplete depletion of TAK1 by siRNA in AGS cells (with ∼80% knockdown efficiency; Fig 2C). The impaired expression of TNF-α and IL-8 could be restored when the siRNA-resistant mouse TAK1 was re-introduced into the knockdown cells (Fig 2D). These data confirm that TAK1 is essential for *H. pylori*-induced activation of NF-κB.

## *H. pylori* stimulates ubiquitination of TAK1

As Lys 63-linked polyubiquitination is important for the activation of TAK1 and IKK (Wang *et al*, 2001; Sorrentino *et al*, 2008), and TAK1 is essential for *H. pylori*-induced activation of NF-κB (Fig 2), we next examined whether *H. pylori* stimulated polyubiquitination of TAK1. Infection of AGS cells with wt, but not *cagA*-deficient, *H. pylori* induced polyubiquitination of endogenous TAK1 (Fig 3A), indicating that CagA is crucial for *H. pylori*-induced polyubiquitination of TAK1.

Next, we determined the potential effect of CagA on the ubiquitination of TAK1. TAK1 is modestly ubiquitinated in the presence of ubiquitin, and this ubiquitination is markedly enhanced by co-transfection of CagA, independently of the tyrosine phosphorylation of CagA (Fig 3B; supplementary Figs S6 and S7 online). The enhanced ubiquitination of TAK1 is Lys 63-linked rather than Lys 48-linked, as enhanced ubiquitination of TAK1 could only be detected in the presence of wt or Lys 63-only
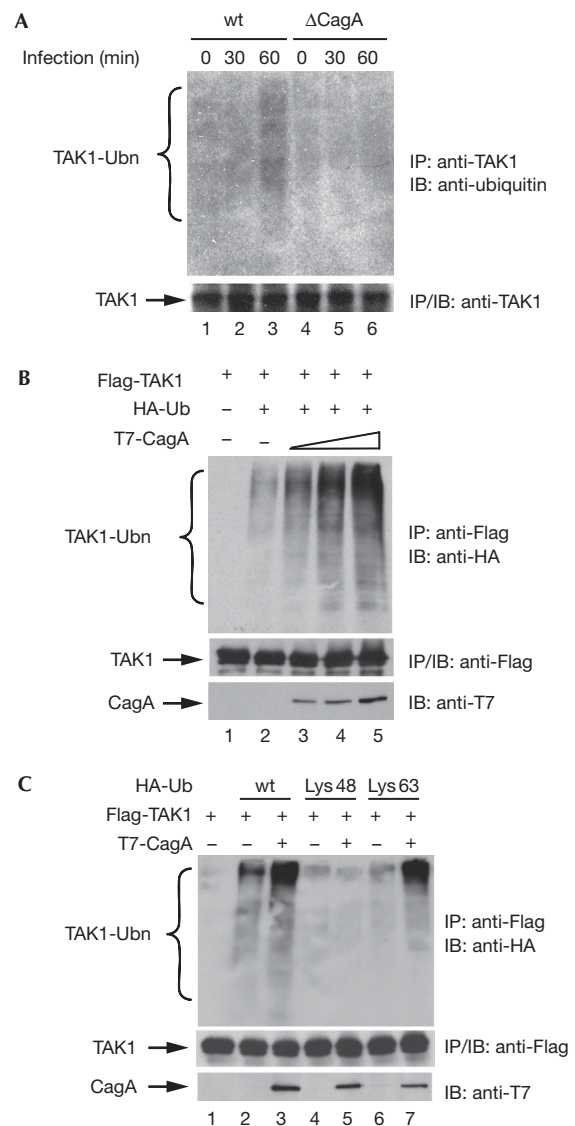
Fig 3 | *Helicobacter pylori* infection induces CagA-dependent TAK1 ubiquitination. (A) Endogenous TAK1 was immunoprecipitated (IP) from AGS cells infected with wt or ΔCagA *H. pylori* and ubiquitination of TAK1 was detected by immunoblotting (IB) with ubiquitin antibodies. **(B)** Flag-TAK1 from HEK293T cells transfected as indicated was immunoprecipitated and immunoblotted for ubiquitination using HA antibodies. **(C)** HEK293T cells were transfected with Flag-TAK1 with or without T7-CagA and with wt, Lys 48-only or Lys 63-only HA–ubiquitin as indicated. Ubiquitination of TAK1 was detected by immunoblotting the TAK1 immunoprecipitates with HA antibodies. HA, haemagglutinin; HEK, human embryonic kidney; TAK1, transforming growth factor-β-activated kinase 1; Ub, ubiquitin; Ubn, ubiquitination; wt, wild type.

ubiquitin, but not in the presence of Lys 48-only ubiquitin (Fig 3C), indicating that ubiquitination of TAK1 enhanced by CagA probably promotes the activity of TAK1 rather than its proteasomal degradation.
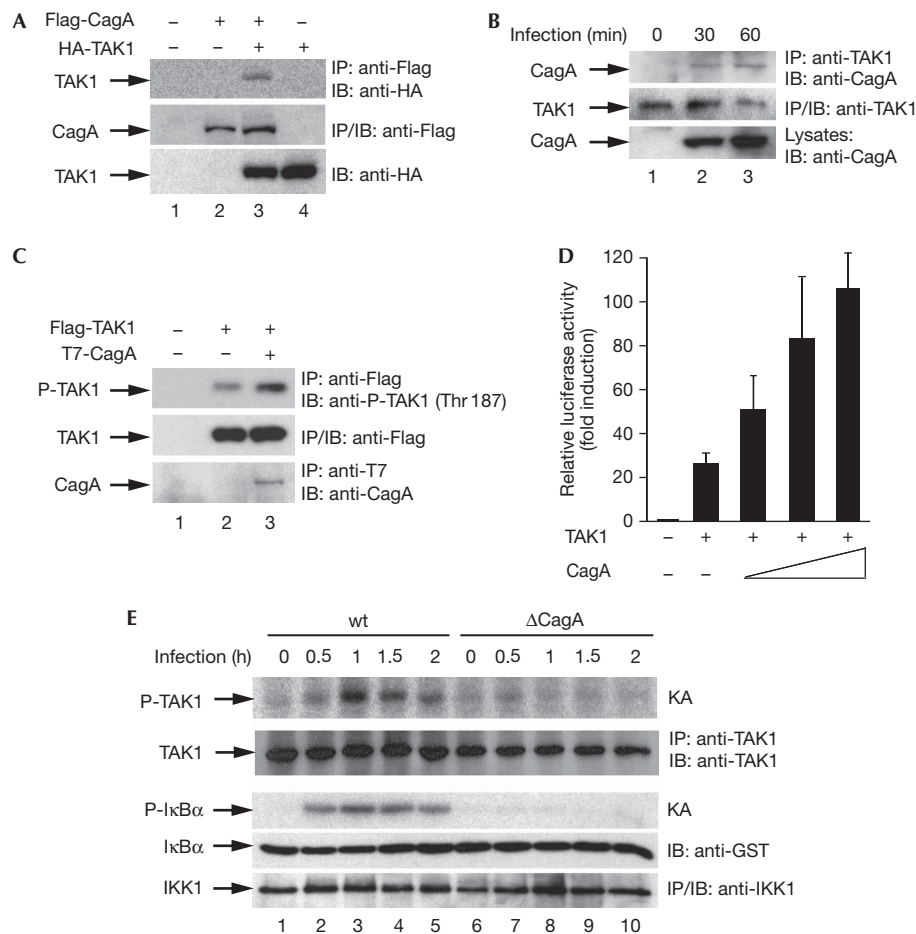
Fig 4 | *Helicobacter pylori* CagA interacts with TAK1 and enhances its activity. (**A**) Ectopically expressed CagA and TAK1 co-immunoprecipitate in HEK293T cells. (**B**) Endogenous TAK1 was immunoprecipitated (IP) from *H. pylori*-infected AGS cells and immunoblotted (IB) for associated CagA with CagA antibodies. (**C**) TAK1 immunoprecipitates from HEK293T cells transfected as indicated were immunoblotted with phospho-TAK1 (Thr 187) antibodies. Levels of immunoprecipitated TAK1 and CagA are shown in the lower panels. (**D**) TAK1 and CagA were co-transfected with an IL-8–luciferase reporter plasmid into HEK293T cells, as indicated. At 30 h after transfection, luciferase activity was measured. (**E**) AGS cells were infected with wt or ΔCagA *H. pylori*. Immunoprecipitates of endogenous TAK1 or IKK were subjected to *in vitro* kinase assays. Kinase activity of TAK1 or IKK was assessed by the auto-phosphorylation of TAK1 immunoprecipitates or phosphorylation of GST–IκBα by IKK1 immunoprecipitates, respectively. GST, glutathione-*S*-transferase; HEK, human embryonic kidney; IKK, IκB kinase; KA, kinase assay; IL-8, interleukin-8; TAK1, transforming growth factor-β-activated kinase 1; wt, wild type.

CagA physically associates with TAK1 *in vitro* and *in vivo*

As CagA often elicits its cellular functions by interacting with cellular proteins (Hatakeyama, 2008), we next assessed the physical interaction between CagA and TAK1. Immunoprecipitation of CagA from transfected human embryonic kidney (HEK) 293T cells co-immunoprecipitated TAK1 (Fig 4A). Furthermore, CagA colocalized with TAK1 on the plasma membrane in transfected AGS cells (supplementary Fig S8 online). An *in vitro* glutathione-*S*-transferase (GST) pull-down assay showed that CagA associated directly with TAK1 through the amino (N)-terminal and carboxy (C)-terminal regions of CagA and the N-terminal region of TAK1 (supplementary Figs S9–S11 online). More importantly, endogenous TAK1 associated with CagA after *H. pylori* infection (Fig 4B). Collectively, these data show that CagA associates with TAK1 *in vitro* and *in vivo* in response to *H. pylori* infection.

CagA enhances the activity of TAK1

As CagA interacts with, and enhances the ubiquitination of, TAK1 (Figs 3 and 4), we next examined whether CagA could activate TAK1. Co-expression of CagA enhanced the kinase activity of TAK1, which is demonstrated by the auto-phosphorylation of TAK1 at Thr 187 (Fig 4C). TAK1-mediated activation of NF-κB was consistently enhanced by CagA in a dose-dependent manner in a κB luciferase reporter assay (Fig 4D). *H. pylori* infection in AGS cells also activated TAK1 and IKK with similar kinetics in a CagA-dependent manner (Fig 4E; supplementary Figs S12 and S13 online). Together, these data indicate that the association of CagA with TAK1 enhances the ability of TAK1 to activate IKK and NF-κB, and that CagA is essential for *H. pylori*-induced TAK1 activation.

CagA is localized to the plasma membrane and undergoes oligomerization—two events that are essential for its signal
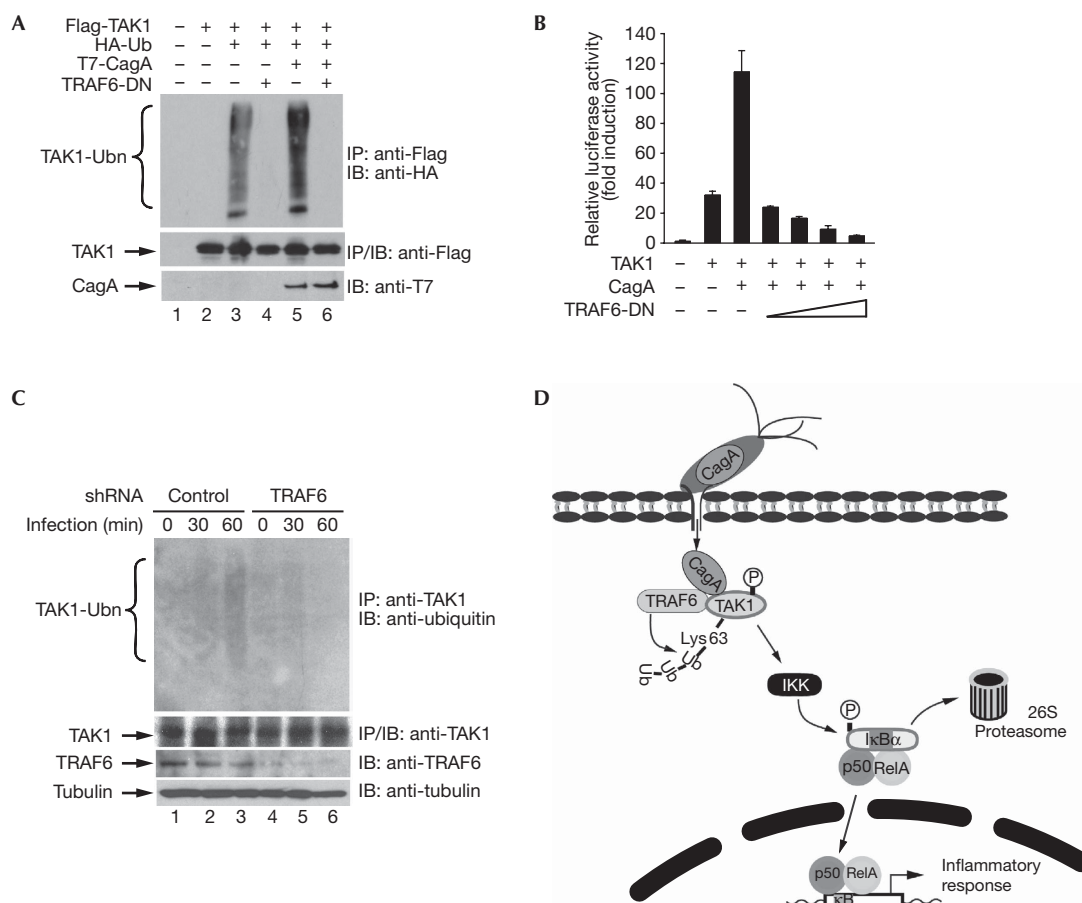
Fig 5 | TRAF6 mediates the ubiquitination and activation of TAK1 by CagA. (A) HEK293T cells were transfected as indicated and TAK1 ubiquitination was detected by immunoblotting (IB) Flag immunoprecipitates (IP) with HA antibodies. **(B)** HEK293T cells were transfected with IL-8–luciferase reporter and other plasmids as indicated. Luciferase activity was measured 30 h after transfection. **(C)** AGS cells stably expressing control or TRAF6 shRNA were infected with *Helicobacter pylori* and ubiquitination of TAK1 was detected by immunoblotting TAK1 immunoprecipitates with ubiquitin antibodies. **(D)** A schematic model illustrating the role of CagA in *H. pylori* infection-induced activation of NF-κB. After injection into cells, CagA associates with TAK1 and TRAF6, and initiates the TRAF6-mediated, Lys 63-linked ubiquitination of TAK1, leading to activation of IKK, NF-κB and the NF-κB-dependent inflammatory response. HA, haemagglutinin; HEK, human embryonic kidney; IL-8, interleukin-8; NF-κB, nuclear factor-κB; shRNA, short hairpin RNA; TAK1, transforming growth factor-β-activated kinase 1; TRAF6-DN, tumour necrosis factor receptor-associated factor 6 dominant-negative; Ub, ubiquitin; Ubn, ubiquitination.

transduction activity (Hatakeyama, 2008). As CagA colocalizes with TAK1 on the plasma membrane (supplementary Fig S8 online), and the oligomerization-defective mutant of CagA fails to induce the ubiquitination of TAK1 or the activation of NF-κB (supplementary Figs S14 and S15 online), the membrane-binding and oligomerization properties of CagA might be important for the activation of TAK1 and NF-κB.

Ubiquitination and activation of TAK1 require TRAF6

As TRAF6-mediated Lys 63-linked ubiquitination is crucial for activation of TAK1 (Wang *et al*, 2001; Sorrentino *et al*, 2008), we next determined the role of TRAF6 in CagA-induced ubiquitination of TAK1. We first assessed the effect of a dominant-negative mutant of TRAF6 (TRAF6-DN; Jono *et al*, 2004) on the polyubiquitination of TAK1. TRAF6-DN not only blocked the ubiquitination of TAK1 but also inhibited the

enhanced ubiquitination of TAK1 facilitated by CagA (Fig 5A). The ability of CagA to enhance TAK1-mediated NF-κB activation was also inhibited by TRAF6-DN (Fig 5B), indicating that ubiquitination of TAK1 is crucial for its activation. Although Lys 34 of TAK1 is ubiquitinated by TRAF6 in response to TGF-β, mutation of Lys 34 to arginine did not affect TAK1 ubiquitination or its ability to activate NF-κB (supplementary Figs S16 and S17 online), indicating that other unidentified lysine residues might also undergo Lys 63-linked ubiquitination, leading to activation of IKK and NF-κB.

To analyse further the role of TRAF6 in *H. pylori*-induced ubiquitination of TAK1 and NF-κB activation, we generated an AGS cell line stably expressing short hairpin RNA (shRNA) against TRAF6 and examined the ubiquitination of TAK1 in these cells. *H. pylori*-induced ubiquitination of TAK1 and mRNA expression of IL-8 and TNF-α were markedly attenuated in the

TRAF6-depleted cells compared with AGS cells stably expressing control shRNA (Fig 5C; supplementary Fig S18 online). These data demonstrate that ubiquitination of TAK1 is crucial for CagA-enhanced NF-κB activation and that TRAF6 is probably the E3 ubiquitin ligase for *H. pylori*-induced TAK1 ubiquitination.

Although CagA enhances TRAF6-mediated Lys 63-linked ubiquitination of TAK1 (Figs 3,5), it is not clear as to how this occurs. CagA fails to enhance the interaction between TRAF6 and TAK1 (supplementary Fig S19 online), excluding the possibility that CagA might function as a bridging factor to facilitate the TAK1–TRAF6 interaction. It is possible that CagA could target TRAF6 and enhance its E3 ligase activity or prevent TAK1 from interacting with deubiquitination enzymes such as CYLD, which inhibit ubiquitination and auto-activation of TAK1 (Reiley *et al*, 2007). Supporting this argument, we found that CagA also interacts with TRAF6 *in vitro* and enhances its auto-ubiquitination (supplementary Figs S20,S21 online). However, binding of TRAF6 to CagA does not seem to affect the interaction between CagA and TAK1 (supplementary Fig S22 online).

In addition to NF-κB, other transcription factors, including AP-1, are activated by *H. pylori* and are involved in the induction of proinflammatory cytokines, including IL-8 (Mitsuno *et al*, 2001; O'Hara *et al*, 2006). Interestingly, AP-1 is also a downstream target of activated TAK1 (Adhikari *et al*, 2007). Further studies are needed to determine whether CagA-enhanced ubiquitination of TAK1 also contributes to *H. pylori*-induced AP-1 activation. It is probable that *H. pylori* uses CagA for activation of several signalling pathways that, in combination, contribute to the ultimate inflammatory response.

In conclusion, our current studies explore the mechanism by which *H. pylori* stimulates NF-κB activation and expression of inflammatory cytokines through the CagA-dependent, TRAF6-mediated Lys 63-ubiquitination and activation of TAK1 (Fig 5D). These studies also contribute to a more comprehensive understanding of how *H. pylori* infection stimulates inflammation and how pathogens, in general, might use cellular signalling molecules to induce cellular responses.

METHODS

Cell lines, *H. pylori* culture and infection. Human AGS gastric adenocarcinoma, HEK293T and MEF cells were cultured in DMEM supplemented with 10% fetal bovine serum. *H. pylori* G27, NCTC11637 and 7.13 strains and their *cagA*-deficient mutants were cultured in bisulphite-free *Brucella* broth on agar media containing Ham's F-12 medium supplemented with 10% fetal bovine serum and 5 μg/ml vancomycin at 37 °C in the presence of 10% CO_2. *H. pylori* was added to AGS or MEF cells for infection at a multiplicity of infection of 50–100. All wt *H. pylori* and the corresponding *cagA*-deficient strains have similar abilities to adhere to the cells.

Immunoprecipitation, immunoblotting analysis and EMSA. Immunoprecipitation and immunoblotting analysis were performed as described previously (Chen *et al*, 2002). For electrophoretic mobility-shift assay (EMSA), whole-cell extracts were collected from infected cells by freeze–thaw lysis (Chen & Greene, 2005) and used for DNA binding assay as described previously (Chen *et al*, 2001).

Quantitative real-time PCR analysis. AGS or MEF cell lines were infected with *H. pylori* for various times and total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). Complementary DNA was synthesized using the Omniscript RT kit (Qiagen). Quantitative real-time PCR was performed using the Qiagen SYBR green PCR kit, with the aid of the 7300 Real-time PCR system (ABI, Foster City, CA, USA). PCR primers for human β-actin, IL-8 and TNF-α, as well as mouse β-actin, E-selectin, IL-6 and TNF-α, were purchased from Qiagen.

Transient transfection and luciferase reporter assay. Transient transfection and luciferase reporter assays were performed as described previously (Chen *et al*, 2002). In each luciferase experiment, cells were also co-transfected with the EF1–*Renilla* luciferase reporter plasmid, which was used as the internal control. Results represent the average of three independent experiments ± s.d.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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