International Journal of Medical Microbiology 303 (2013) 548–552

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

Short communication

Helicobacter pylori induces type 4 secretion system-dependent, but CagA-independent activation of IκBs and NF-κB/RelA at early time points

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ARTICLE INFO

Article history:
Received 30 April 2013
Received in revised form 4 July 2013
Accepted 20 July 2013

Keywords:
*cag*PAI pathogenicity island
IκBs
Inflammation
RelA phosphorylation

ABSTRACT

Colonization of the gastric epithelium by *Helicobacter pylori* induces the transcription factor nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB) and the innate immune response. Virulent strains of *H. pylori* carry a *cag* pathogenicity island (*cag*PAI), which encodes a type IV secretion system (T4SS). Recent publications have shown controversial data regarding the role of the T4SS and the effector protein cytotoxin associated gene A (CagA), which becomes translocated by the T4SS into the eukaryotic epithelial cell, in *H. pylori*-induced NF-κB activation. Thus, this study analyses by using three different *H. pylori* strains (*P1, B128 and G27)* whether CagA is required to initiate activation of different molecules of inhibitors of kappa B (IκB) and the NF-κB transcription factor RelA. We provide experimental evidence that *H. pylori* induces phosphorylation of NF-κB inhibitors IκBα, IκBβ and IκBε, and degradation of IκBα. Further, *H. pylori* stimulates phosphorylation of RelA at amino acids S536, S468 and S276, promotes DNA binding of RelA, and interleukin 8 (IL-8) gene expression in a T4SS-, but CagA-independent manner at early time points.

Introduction

The microaerophilic bacterium *H. pylori* induces gastric inflammation which could promote peptic ulceration and progression to gastric cancer (McLean and El-Omar, 2011). Infection of the gastric mucosa with *H. pylori* induces the release of chemotactic cytokines including IL-8 (Gerhard et al., 2002). The IL-8 promoter is predominantly regulated by NF-κB. Regulation of NF-κB activity by *H. pylori* is a fast process involving phosphorylation of the NF-κB inhibitor IκBα at serine residues by IκB kinase β (IKKβ) (Foryst-Ludwig and Naumann, 2000; Hirata et al., 2006). Phosphorylation creates a recognition signal for ubiquitinylating enzymes, thereby marking IκBα for proteasomal degradation. This liberates the NF-κB dimer, *e.g.* RelA/p50, which translocates to the nucleus and binds to the promoter of target genes, such as *il-8* and *iκbα* (Neish and Naumann, 2011).

It has been shown that *H. pylori*-induced IL-8 release and NF-κB activity require a functionally active T4SS, but not CagA (Sharma et al., 1998; Neu et al., 2002; Foryst-Ludwig et al., 2004; Hirata et al., 2006; Schweitzer et al., 2010). The role of CagA in the regulation of NF-κB activity and, therefore, in induction of the inflammatory response, is still a subject of intense discussion (Backert and Naumann, 2010). CagA-dependent release of IL-8 at late time points has been shown previously (Brandt et al., 2005; Suzuki et al., 2009). Further, it has been suggested that *H. pylori* CagA is required for IκBα degradation and RelA activation (Lamb et al., 2009).

Our work shows, by studying in parallel different *H. pylori* strains and their CagA-deficient mutants, that in contrast to the functional T4SS, the CagA protein is dispensable for NF-κB activation at early time points.

Materials and methods

Cell culture and bacteria

AGS (ATCC) cell line was maintained in RPMI 1640 medium (PAA Laboratories) supplemented with 10% fetal bovine serum (FBS) and

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http://dx.doi.org/10.1016/j.ijmm.2013.07.008

penicillin/streptomycin at 37° C in a humidified 5% CO_2 atmosphere. Sixteen hours prior to stimulation, the cell medium was replaced with antibiotics-free RPMI 1640 supplemented with 0.5% FBS.

H. pylori strains P1 (wt, *cagA*, *virB7*) (Backert et al., 2000), B128 (wt, *cagA*, *virB10*) (Rieder et al., 2005) and G27 (wt, *cagA*) (Censini et al., 1996) were constructed as described previously and plated onto GC agar supplemented with horse serum, vancomycin, trimethoprim, nystatin and vitamins. The bacterial colonies grew in standard microaerophilic conditions for 48–72 h, and then bacterial suspensions in PBS were prepared. *H. pylori* was added to AGS cells at a multiplicity of infection of 100.

Recombinant human TNFα (R&D Systems) was used in a concentration of 10 ng/ml.

Immunoblots

Cell lysates were prepared with a modified RIPA buffer as described previously (Sokolova et al., 2008). The lysates were boiled with the Laemmli buffer for 5 min, and equal amounts of protein extracts were separated by SDS-PAGE and electrotransferred onto Immobilon-P transfer polyvinylidene fluoride membranes (Millipore). The blots were incubated with primary antibodies diluted in 5% non-fat milk overnight at 4° C and subsequently with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories). Immunoblots were developed using the enhanced chemiluminescence detection kit Amersham™ ECL™ (GE Healthcare). The following primary antibodies were used: mouse anti-*H. pylori* flagellin (Acris GmbH), -CagA (Austral Biologicals), -NF-κBp65 (BD Biosciences Pharmingen), -phospho-IκBα (Ser32/36) (5A5) (Cell Signaling Technology Inc.), -GAPDH (Millipore), and rabbit anti-IκBα (44D4), -phospho-IκBβ(T19/S23), -phospho-IκBε(S18/22), -phospho-NF-κB p65(S536), -phospho-NF-κB p65(S276), -phospho-NF-κB p65(S468) (Cell Signaling Technology Inc.).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared using a nonionic detergent method as described (Naumann and Scheidereit, 1994). An annealed oligonucleotide containing the Igκ-binding site was labeled using the T4 polynucleotide kinase in the presence of [γ-^{32}P] dATP. The DNA-binding reaction was performed in 20 μl buffer (2 μg poly(dI-dC), 1 μg BSA, 5 mM DTT, 60 mM KCl, 10% glycerol and 20 mM HEPES, pH 8.4) for 20 min at 30° C. For the identification of the proteins in the NF-κB heterodimer an anti-RelA antibody was added to the lysate. The oligonucleotide/protein complexes were separated by native electrophoresis using 5% polyacrylamide gel. After drying, the gel was exposed to Amersham TM film (Amersham Pharmacia Biotech) at −80 °C using an intensifying screen.

RNA isolation and RT-PCR

Total RNA was extracted with the RNeasy Plus Micro kit (Qiagen). cDNA was synthesized from 1 μg of RNA using a random hexamer primer and RevertAid™ First Strand cDNA Synthesis kit (Fermentas). cDNA was amplified using SensiMix DNA kit (Quantace) in the StepOne™ cycler (Applied Biosystems) as described (Sokolova et al., 2008) using the following primers:

5′-AGATGTCAGTGCATAAAGACA-3′ (forward);
5′-TATGAATTCTCAGCCCTCTTCAAAAA.-3′ (reverse) for IL-8; and
5′-TCCAAAATCAAGTGGGGCGATGCT-3′ (forward);
5′-CCACCTGGTGCTCAGTGTGACCC-3′ (reverse) for GAPDH.

Densitometric quantification

Quantification of proteins as fold changes of band intensities relative to non-stimulated cells (0 min control) was performed using VersaDoc Imaging System and Quantity One software (Bio-Rad). Local background subtraction was applied.

Statistical analysis

Statistical analysis of the results was performed using the Student's *t*-test. The data are expressed as the mean fold changes from a triplicate ± SEM with the value of the control arbitrarily normalized to 1.

Results and discussion

In this study we investigated the impact of the T4SS and the effector protein CagA from different *H. pylori* strains (P1, G27 and B128) on NF-κB activation in AGS cells. A similar abundance of the flagellin protein in lysates of infected cells indicated that bacterial adherence of *H. pylori* P1 wt strain and its isogenic mutants (*cagA* or *virB7*) to AGS cells was equal (Fig. 1A). The same number of bacteria of the isogenic strains exhibited similar amounts of flagellin (Fig. 1B). Further, there was no difference in the amount of CagA between the wt and VirB7-deficient bacteria (Fig. 1A and B). The VirB7 protein is required for the assembly of the pilus-like T4SS (Tanaka et al., 2003; Fischer, 2011). Infection with the wt and *cagA*, but not with the *virB7* strain led to transient phosphorylation of IκBα, IκBβ and IκBε. IκBα degradation was detected within 15 min post infection (p.i.). *De novo* synthesized IκBα was observed within 60 min p.i. Phosphorylation of IκBα was sustained for the entire infection kinetics, whereas IκBβ and IκBε phosphorylation was transient (Fig. 1A). Phosphorylation of IκBα on two conserved residues S32 and S36 has been shown to promote K48-linked ubiquitinylation of the protein at K21 and K22 by a Skp1/Cul1/F-box protein (SCF) cullin-RING ubiquitin-ligase (CRL) leading to subsequent degradation of IκBα by the 26S proteasome (Perkins, 2007). In many cells, nearly half of the cytosolic NF-κB is sequestered by IκBβ and IκBε, which efficiently mask the NF-κB nuclear localization sequence (Tam and Sen, 2001). We show here for the first time that *H. pylori* induces phosphorylation of IκBβ and IκBε in AGS cells. Thus, phosphorylation of IκBβ and IκBε, which leads to their ubiquitinylation and degradation similarly to IκBα, efficiently contributes to T4SS-dependent regulation of NF-κB. Additionally, the wt and CagA-deficient *H. pylori* stimulated multiple phosphorylations of RelA (S536, S468 and S276). Phosphorylation of RelA at amino acids S536 and S468 was fast (within 15 min), and phosphorylation at S276 was slightly delayed (Fig. 1A). TNFα, as a positive control, also induced phosphorylation of RelA and IκBs (Fig. 1C). Nuclear translocation of NF-κB is accompanied by phosphorylation of RelA (Neumann and Naumann, 2007). In *H. pylori*-infected cells, the phosphorylation of S276 and S468 in RelA has not been previously described. Phosphorylation of RelA at S276, which enhances RelA binding to DNA and promotes transcriptional activity by recruiting the co-activator CBP/p300, could be directed by protein kinase A (PKA) and mitogen-activated protein kinases (MAPK). PKA phosphorylates RelA most likely after its activation, which can explain a delayed phosphorylation at S276 upon infection. Phosphorylation of S468 could be mediated by glycogen synthase kinase 3 β (GSK3β), IKKβ or IKKε, and the main activating phosphorylation at S536 could be directed by a number of kinases including IKKβ (Neumann and Naumann, 2007).

H. pylori-induced IκB phosphorylation and IκBα degradation allowed nuclear translocation and binding of NF-κB to promoter elements. The wt and *cagA* strains induced strong binding of

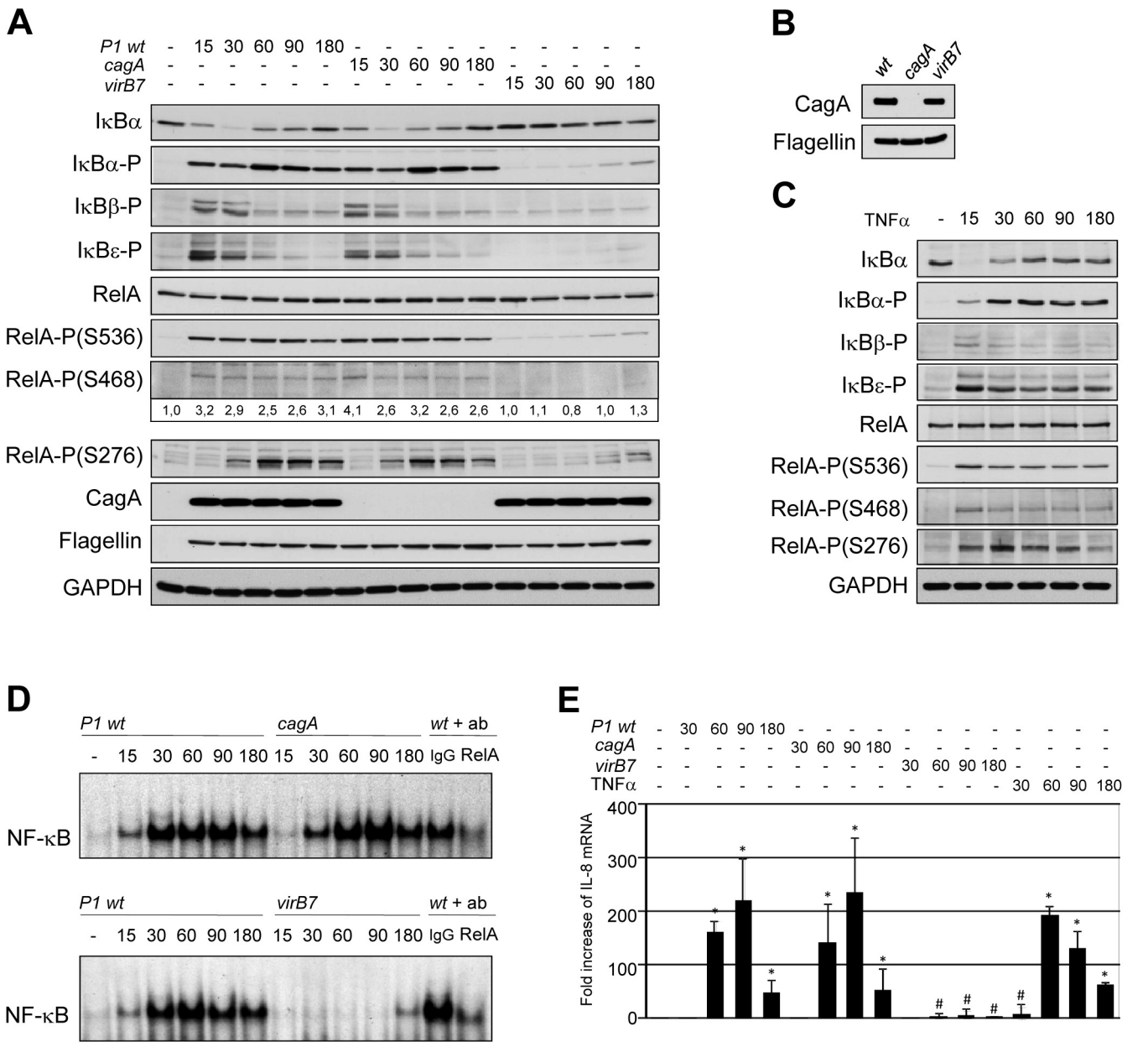

Fig. 1. *H. pylori* P1 regulates NF-κB/RelA and IκBs in a T4SS-dependent manner. Cells were infected with *H. pylori* P1 wt strain, the *cagA* or *virB7* mutants, or were stimulated as positive control with TNFα for the indicated periods of time. Immediately after inoculation of the bacteria, cell culture plates were centrifuged for 3 min to achieve an equal settling of the wt and the mutants to the eukaryotic cells. (A and C) AGS cell lysates were analyzed by immunoblotting using antibodies against the indicated proteins. Immunostaining of RelA and GAPDH were performed to show equal protein loading. As expected, CagA was detected only in lysates of the wt- and *virB7*-infected cells, but not in lysates of the *cagA*-infected cells. Densitometric quantification is shown for RelA(S468) phosphorylation. (B) An equal number of bacteria of the P1 wt strain, the *cagA* or *virB7* mutants were lysed and analyzed by immunoblotting using antibodies against flagellin and CagA. (D) Nuclear extracts of *H. pylori*-infected AGS cells were prepared and subjected to EMSA as described in Materials and methods. (E) Total RNA was isolated and IL-8 expression was analyzed by qRT-PCR. $^{*}P < 0.01$, compared with non-stimulated cells, $^{\#}P < 0.01$ compared with wt-stimulated cells.

NF-κB to an oligonucleotide containing the Igκ-binding site (Fig. 1D). We detected also weak NF-κB DNA binding by the *virB7* strain at 180 min (Fig. 1D), which was consistent with the phosphorylation of IκBα and RelA(S276) at 180 min (Fig. 1A). A further increase of NF-κB activity after 180 min was not observed (data not shown). We think that unknown cellular processes induced by *H. pylori* in a T4SS-independent manner could lead to a weak activation of NF-κB. It would be interesting to investigate this issue in future work. *H. pylori*-induced NF-κB binding to DNA was reduced when nuclear extracts were incubated with an anti-RelA antibody, whereas a preimmune serum (IgG) did not show the reduction, indicating that the nuclear NF-κB heterodimer contained RelA (Fig. 1D). Further, stimulation of AGS cells with TNFα or infection with *H. pylori* wt or the *cagA*, but not with the *virB7* mutant led to a strong accumulation of IL-8 mRNA following the NF-κB-driven induction of the IL-8 gene promoter with a maximal effect within 90 min (Fig. 1E). Thus, a functionally intact T4SS, but not CagA is required for *H. pylori*-induced IL-8 mRNA production at early time points.

A CagA-deficient mutant of the B128 strain was as potent as the wt B128 strain in inducing phosphorylation and degradation of IκBα, and phosphorylation of RelA (S276 and S536) in AGS cells. In contrast to the *cagA* strain, the mutant *virB10* strain deficient in the T4SS core protein VirB10 did not influence IκBα stability and RelA

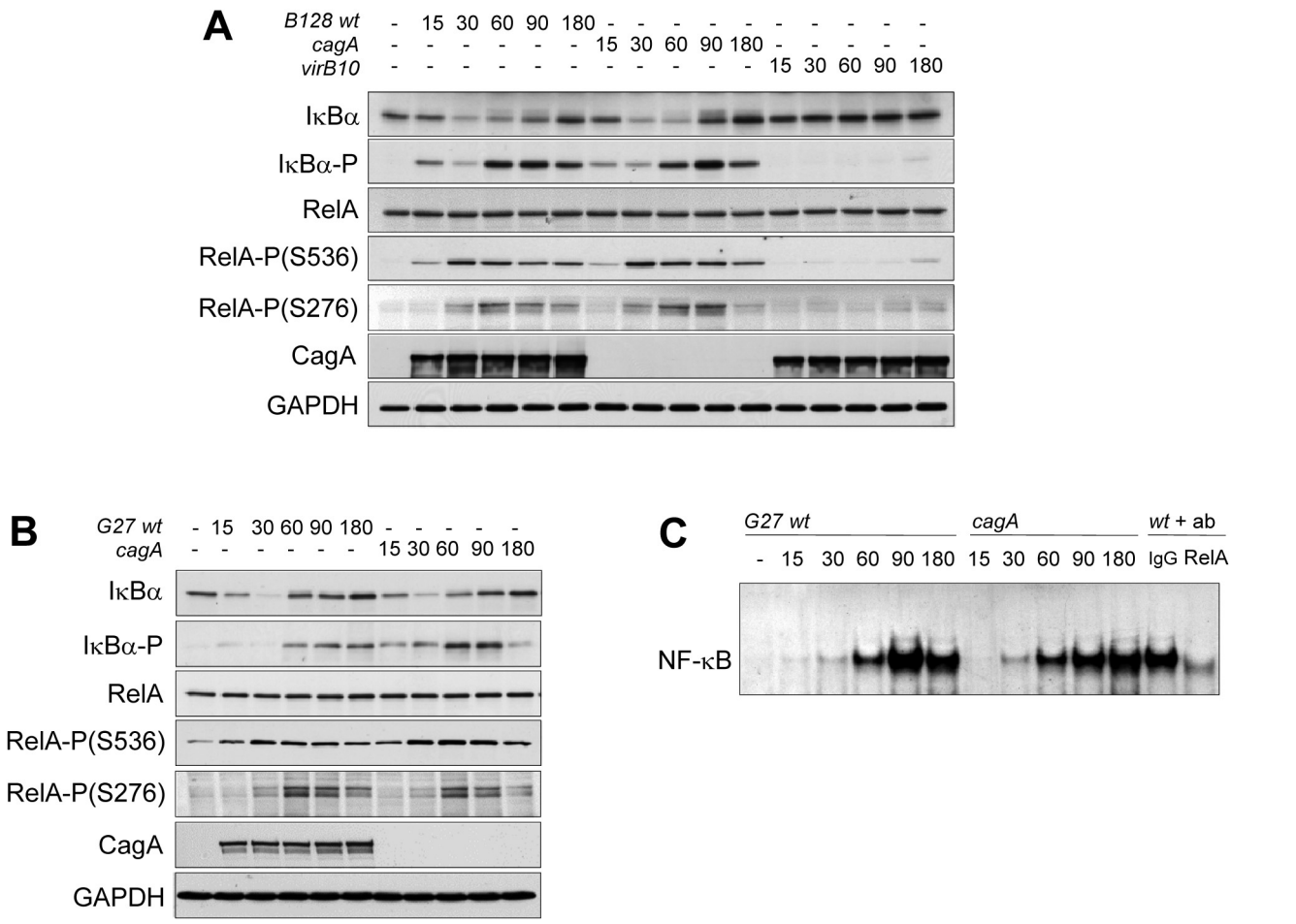

Fig. 2. T4SS-dependent regulation of NF-κB/RelA and IκBs by *H. pylori* B128 and G27 strains. (A and B) AGS cells were infected with different *H. pylori* wt strains and mutants as indicated, and cell lysates were prepared at the indicated periods of time. Cell lysates were analyzed by immunoblotting using the antibodies against the indicated proteins. Immunostainings of RelA and GAPDH were performed to show equal protein loading. (C) Nuclear extracts of *H. pylori* G27-infected AGS cells were prepared and subjected to EMSA as described in Materials and methods.

phosphorylation (Fig. 2A), indicating that the integrity of the T4SS is required for activation of NF-κB. In addition, heat-inactivated bacteria were not capable to induce IκBα phosphorylation (data not shown) indicating that the viability of *H. pylori* is a prerequisite for NF-κB activation in epithelial cells.

Further, *H. pylori* strain G27 wt and its isogenic CagA-deficient mutant induced degradation and phosphorylation of IκBα, as well as a strong transient increase of RelA phosphorylation (S276 and S536) in AGS cells (Fig. 2B). The wt and *cagA* G27 strains stimulated formation of RelA-DNA complexes in the nucleus (Fig. 2C), which has been shown previously by Neu et al. (2002). In contrast to these data, Lamb et al. (2009) have suggested that NF-κB is not activated by the CagA-deficient mutant of the G27 strain. Based on the data by Neu et al. (2002) and our data using three different *cagA*-deficient *H. pylori* mutants (including the G27 strain), which rapidly activate NF-κB similar to the wt strains favor that the T4SS, but not CagA is required for *H. pylori*-induced NF-κB activation at early time points. Additional work regarding the identification of regulatory factors further upstream in NF-κB signaling may help to resolve the discrepancy in published data.

Acknowledgements

We thank K. Guillemin for G27 and R. Haas for B128 strains of *H. pylori*. The work was supported in part by the German Research Foundation (SFB854 TP4 and GRK1167) by grants to M.N.

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