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Phosphorylation of tyrosine 972 of the *Helicobacter pylori* CagA protein is essential for induction of a scattering phenotype in gastric epithelial cells

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Summary

Helicobacter pylori **colonizes the human stomach and is the causative agent of a variety of gastric diseases. After bacterial attachment, the *H. pylori* CagA protein is translocated into gastric epithelial cells and tyrosine phosphorylated. This process is associated with characteristic cytoskeletal rearrangements, resulting in a scatter factor-like ('hummingbird') phenotype. In this study, using a *cagA* mutant complemented with wild-type *cagA* and transiently expressing CagA in AGS cells, we have demonstrated that translocated CagA is necessary for rearrangements of the actin cytoskeleton to occur. Anti-phosphotyrosine immunoblotting studies and treatment of infected cells with phosphotyrosine kinase inhibitors suggested that not only translocation but also phosphorylation of CagA is important in this process. Transient expression of CagA–green fluorescent protein (GFP) fusion proteins and two-dimensional gel electrophoresis of CagA protein species demonstrated tyrosine phosphorylation in the C-terminus. Site-directed mutagenesis of CagA revealed that tyrosine residue 972 is essential for induction of the cellular phenotype. We have also demonstrated that translocation and phosphorylation of CagA is necessary but not sufficient for induction of the hummingbird phenotype in AGS cells, indicating the involvement of as yet unidentified bacterial factor(s).**

Introduction

Direct injection of virulence factors by bacteria into host cells has been described for two types of secretion machinery, termed type III and type IV (Cornelis and Wolf-Watz, 1997; Finlay and Falkow, 1997; Covacci *et al*., 1999; Galan and Collmer, 1999; Christie and Vogel, 2000). Model systems have been established for enteropathogenic *Escherichia coli* (EPEC), *Salmonella*, *Shigella and Yersinia* (type III) or *Agrobacterium tumefaciens*, *Bordetella pertussis*, *Legionella pneumophila* and *Helicobacter pylori* (type IV). These two secretion systems are functionally equivalent, but developed from a different ancestor during evolution. In most of the systems, effector proteins that are unique for each bacterial species are delivered from the bacterium into the host cell. The prototypical type IV system is the *A. tumefaciens* transfer (T)-DNA transfer machine, which delivers oncogenic nucleoprotein particles into plant cells (e.g. Pansegrau and Lanka, 1996; Christie and Vogel, 2000). Pathogens specialized in type III secretion target various components of eukaryotic signal transduction pathways in order to attach to or enter into host cells or to block bacterial uptake by phagocytosis (e.g. Cornelis and Wolf-Watz, 1997; Finlay and Falkow, 1997; Kenny *et al*., 1997; Galan and Collmer, 1999). Although many of the components of the type III secretory pathways have been characterized, very little is known about the type IV secretion machineries and their effector proteins targeted into mammalian host cells. Understanding the biology of these secretion systems may allow the development of novel therapeutic approaches for several infectious diseases.

Helicobacter pylori is a highly successful bacterial pathogen that colonizes the human stomach. This bacterium has been recognized as the causative agent of chronic gastric inflammation, which can progress further to a variety of diseases such as peptic ulcer and mucosa-associated lymphoid tissue (MALT) lymphoma or adenocarcinoma (Covacci *et al*., 1999; Cover and Blaser, 1999). Type I isolates of *H. pylori* encode a major disease-associated genetic component, the *cag* pathogenicity island (PAI). *H. pylori* type II strains lack the entire *cag* PAI and resemble commensal bacteria rather than pathogens (Covacci *et al*., 1999). Genetic and functional studies have provided evidence that *cag* PAI encodes a type IV secretion apparatus for the delivery of virulence factors such as the immunodominant CagA protein (Censini *et al*., 1996; 2001; Akopyants *et al*., 1998). Six of the *H. pylori cag* PAI genes are homologous to the well-known virulence genes *virB4*, *virB7*, *virB8*, *virB9*, *virB10*, *virB11* and *virD4* from type IV transporter systems of *A. tumefaciens*, *B. pertussis* or *L. pneumophila* (Pansegrau

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and Lanka, 1996; Akopyants *et al*., 1998; Covacci *et al*., 1999; Christie and Vogel, 2000; Censini *et al*., 2001). The crystal structure of the *H. pylori* traffic ATPase VirB11 revealed six monomers in two ring-shaped core complexes, which are proposed to function as an ATP-regulated inner membrane pore (Yeo *et al*., 2000). Very recently, we and other laboratories have collectively demonstrated that CagA is translocated from the bacterium into the host cell (Segal *et al*., 1999; Asahi *et al*., 2000; Backert *et al*., 2000; Odenbreit *et al*., 2000; Stein *et al*., 2000). CagA secretion depends on the expression of functional genes of the VirB/D complex encoded by the *cag* PAI.

In vitro studies have shown that infection with *cag* PAI-positive *H. pylori* strains induced the reorganization of the host cell actin cytoskeleton (Segal *et al*., 1996), the activation of Rho GTPases Rac1 and Cdc42 (Churin *et al*., 2001), the recruitment of transcription factors NF-κB (Keates *et al*., 1997; Münzenmaier *et al*., 1997) and AP-1 (Naumann *et al*., 1999), the activation of proto-oncogenes c-fos and c-jun (Meyer-ter-Vehn *et al*., 2000) and the release of cytokines and chemokines (Tummuru *et al*., 1995; Censini *et al*., 1996; Bodger and Crabtree, 1998; Li *et al*., 1999; Yamaoka *et al*., 2000). However, the role of CagA in these events remains unclear. In infected gastric epithelial cells (Backert *et al*., 2001) and phagocytes (Moese *et al*., 2001; Odenbreit *et al*., 2001), CagA was partially found to be processed into a 100–105 kDa fragment ($p100^{CagA}$) and a 30–40 kDa fragment ($p35^{CagA}$), but the importance of this event is not understood. Peptides from the C-terminus of CagA were identified by MALDI-mass spectrometry in $p35^{CagA}$ purified from infected U937 cells (Moese *et al*., 2001). Once translocated into eukaryotic cells, CagA is tyrosine phosphorylated by an as yet unknown host kinase (Segal *et al*., 1999; Asahi *et al*., 2000; Backert *et al*., 2000; Odenbreit *et al*., 2000; Stein *et al*., 2000). Furthermore, the relative importance of the two or three potential tyrosine phosphorylation sites in different CagA variants (Asahi *et al*., 2000; Backert *et al*., 2000; 2001; Odenbreit *et al*., 2000) remains unknown. Deconvolution and confocal immune fluorescence microscopy demonstrated that bacteria attached to the cell surface were associated with phosphorylated CagA in the host cell (Segal *et al*., 1999; Backert *et al*., 2000; Odenbreit *et al*., 2000). This process was accompanied by changes in cell morphology, such as elongation and spreading, leading to the formation of the so-called 'hummingbird' phenotype, the effacement of microvilli and pedestal formation at the attachment site (Segal *et al*., 1996; 1999).

In most of the *in vivo* and *in vitro* infection studies performed with *H. pylori* so far, *cag* PAI mutants have been used, in which the polar effects of integrated resistance gene cassettes on the expression of flanking genes of the type IV transporter complex were not investigated. Complementation or transient expression of individual *cag* PAI genes has not been carried out so far. Therefore, the role of CagA during infection is still unclear. In the present report, we studied tyrosine phosphorylation and the function of CagA using CagA of strain 26695 (Tomb *et al*., 1997) and the gastric epithelial adenocarcinoma cell line AGS as a model system. We complemented our *cagA* mutant with intact *cagA in trans* and produced a set of *cagA* constructs with single amino acid substitutions. We present several lines of evidence that the phosphorylation of tyrosine residue 972 (Y-972) on the CagA protein is essential for the host signalling response, leading to the induction of the scatter factor-like ('hummingbird') phenotype in gastric epithelial cells.

Table 1. Phosphorylation of CagA and expression of a scattering ('hummingbird') phenotype in AGS cells infected with H. pylori.*

H. pylori strain	Strain number	Mutated gene	CagA translation	CagA phosphorylation	Induction hummingbird phenotype
P1	P1	–	+	+	+
P12	P12	–	+	+	+
J99	P212	–	+	–	–
26695	P225	–	+	+	+
P227	P227	–	+	+	+
1061	P228	–	–	–	–
G27	P229	–	+	+	+
P12	P14	*vacA*	+	+	+
P1	P205	*virB4*	+	–	–
P1	P206	*virB7*	+	–	–
P1	P207	*virB10*	+	–	–
P1	P208	*virB11*	+	–	–
P1	P209	*virD4*	+	–	–
P1	P211	*cagA*	–	–	–
P1Δ*cagA*/*cagA*	P230	*cagA*	+	+	+

* All *H. pylori* strains are type I isolates, except P228, which is a type II strain lacking the *cag* PAI. P230 is a P1Δ*cagA* mutant strain complemented with shuttle vector pHel2 expressing intact CagA of strain 26695. Bacterial infection was for 4 h using a MOI of 100. CagA translation and tyrosine phosphorylation was detected by immunoblot analysis.

Results

Induction of a scattering phenotype in infected target cells depends on the H. pylori *CagA protein and correlates with CagA tyrosine phosphorylation*

AGS gastric epithelial cells were infected with the *H. pylori* strains listed in Table 1. The morphological changes associated with the scatter factor-like ('hummingbird') phenotype, characterized by spreading and elongation of the host cell, the presence of lamellipodia (thin actin sheets at the edge of the cell) and filopodia (spikes containing a tight bundle of actin filaments) (Segal *et al*., 1999), were induced by *H. pylori* type I strains but not by type II strains or isogenic mutants of individual *vir* or *cagA* genes of the *cag* PAI (Fig. 1, Table 1). To exclude polar effects in our P1Δ*cagA* mutant and to demonstrate that CagA was directly involved in the induction of the hummingbird phenotype, we complemented the P1Δ*cagA* mutant with intact *cagA* expressed from the shuttle vector pHel2 (P1Δ*cagA/cagA*). Infection of AGS cells with this *H. pylori* strain did indeed restore the formation of the cellular phenotype (Fig. 1, bottom). Treatment of AGS cells with the tyrosine kinase inhibitor genistein followed by infection with P1Δ*cagA/cagA* prevented the induction of cytoskeletal rearrangements, suggesting that tyrosine phosphorylation is essential for this process (Fig. 1, bottom). The hummingbird phenotype was observed to be time and dose dependent and occurred in 65–80% of the AGS cells as soon as 3–4 h after bacterial attachment (Fig. 2A and B) using multiplicities of infection (MOIs) of 25, 50, 100 or 150 (data not shown). The needle-like structures formed during infection were 25–70 μm in length (double arrows), thus exceeding the size of uninfected AGS cells up to four times (Fig. 2A). Occasionally, elongated AGS cells were also seen in the controls (infection with *cag* PAI mutants or uninfected cells), but their number was usually $< 5\%$ with only short (< 20 μm) needle-like protrusions (Fig. 2A and B). Anti-phosphotyrosine immunoblotting experiments demonstrated that the induction of this phenotype did indeed correlate with the presence of tyrosine-phosphorylated CagA (Figs 2B, bottom, 2C and Table 1). Collectively, these findings provided final evidence that: (i) our P1Δ*cagA* mutant did not affect the expression of flanking genes of the type IV secretion system encoded in the *cag* PAI; (ii) CagA is involved in actin cytoskeletal rearrangements of the host cell (scattering phenotype); and (iii) phosphorylation of CagA might be important in this process.

Tyrosine phosphorylation of CagA occurs at the C-terminus of the protein

In order to understand the role of the CagA protein in the infection process and the induction of the hummingbird phenotype, we examined tyrosine phosphorylation of

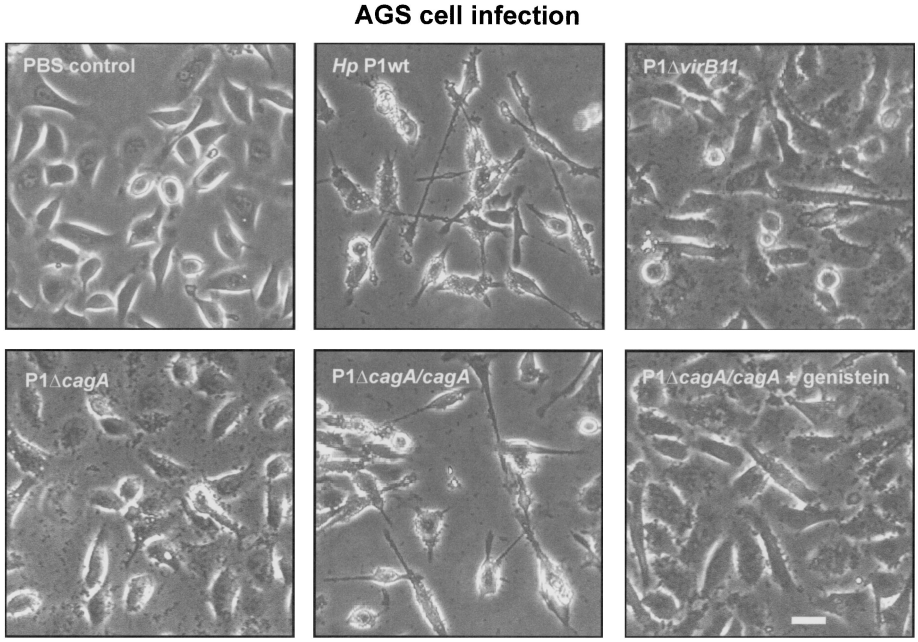


Fig. 1. Induction of the scattering ('hummingbird') phenotype in AGS cells by *H. pylori* depends on functional *virB11 and cagA* genes. Phase contrast micrographs of AGS cells infected with wild-type strain P1 and isogenic mutants of the type IV secretion apparatus encoded by the *cag* PAI are shown. Infection with P1Δ*cagA* complemented with intact CagA expressed from shuttle vector pHel2 (P1Δ*cagA/cagA*) restored the hummingbird phenotype. This phenotype was not observed in the presence of 250 μM genistein, a well-known tyrosine kinase inhibitor. In each experiment *H. pylori* infection was for 4 h using a MOI of 100. Scale bar = 10 μm.

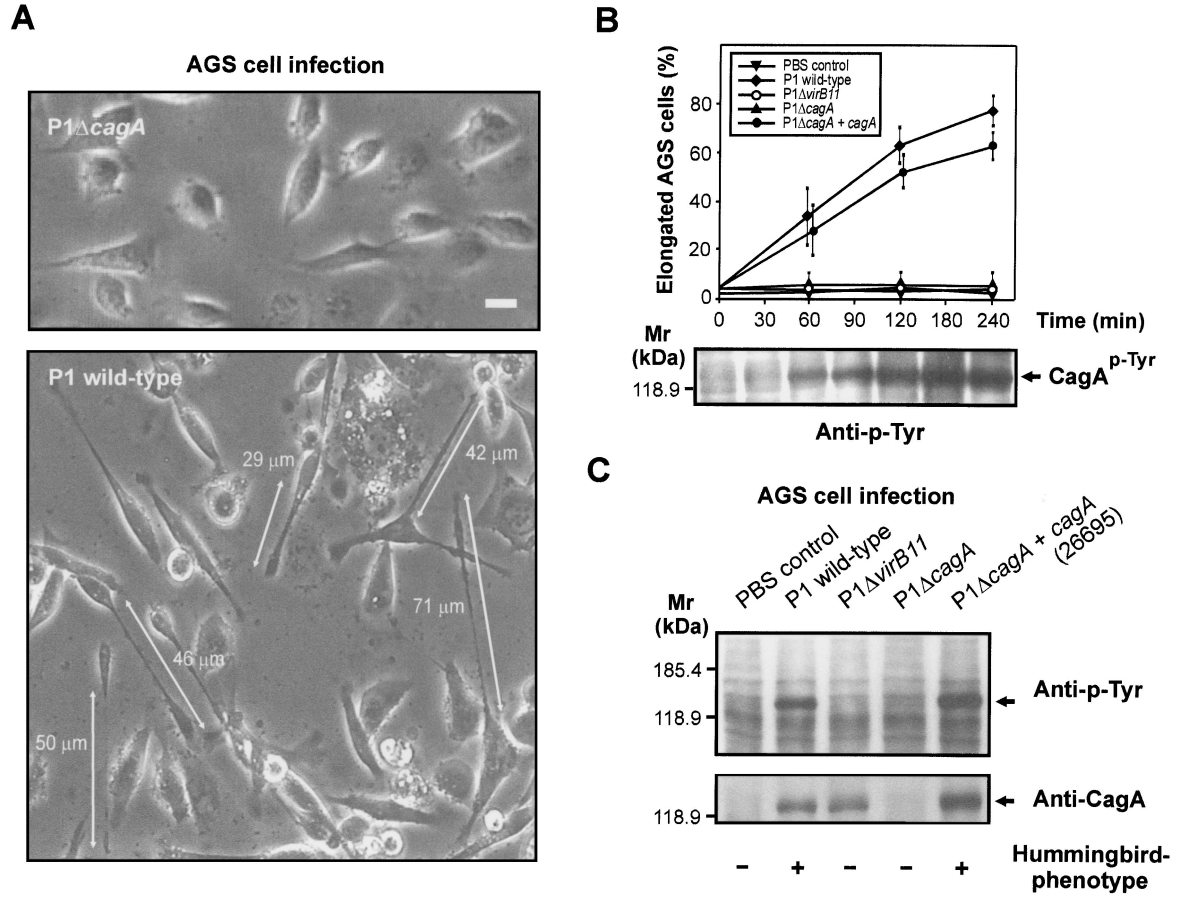
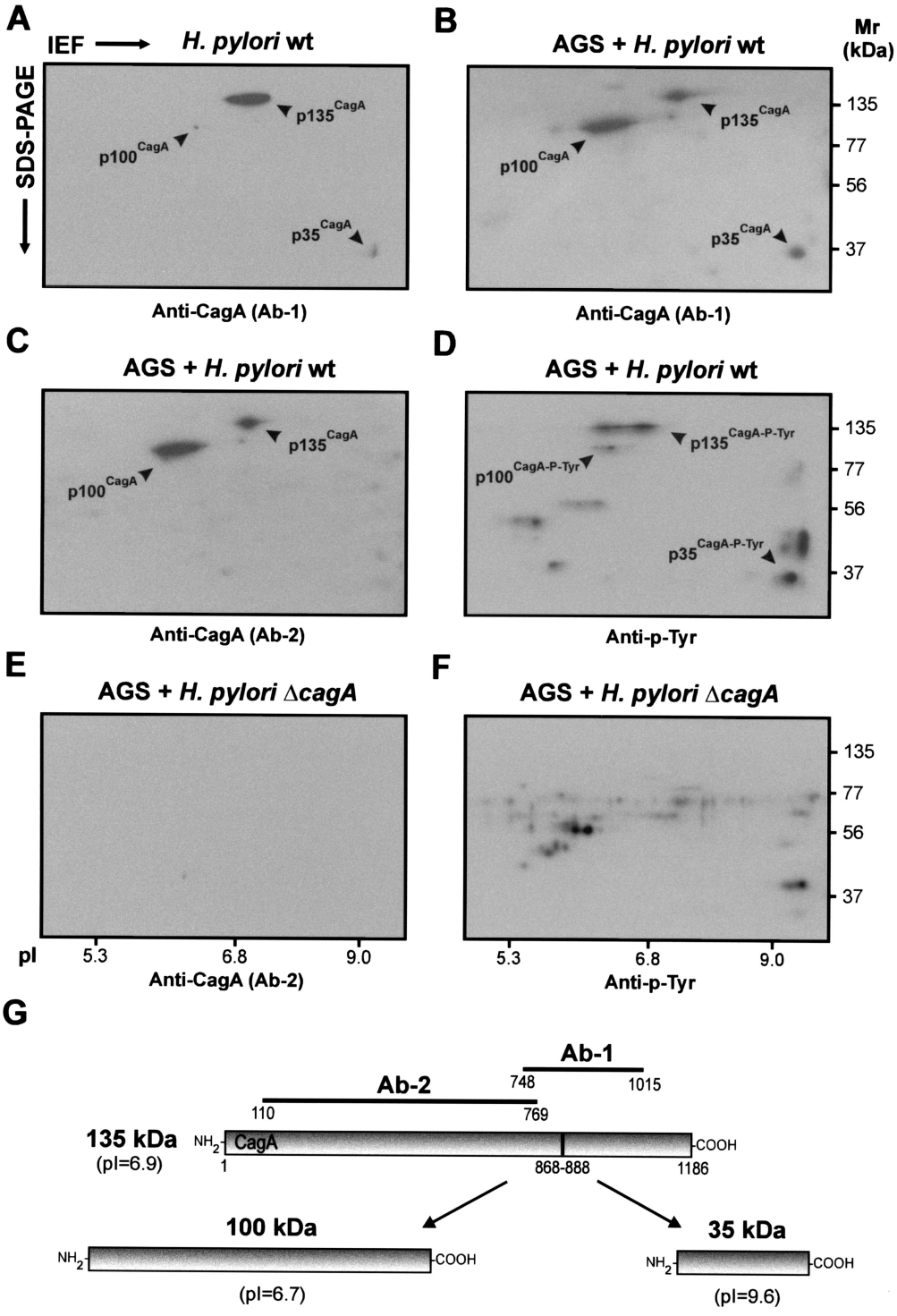


Fig. 2. Correlation between induction of the scattering ('hummingbird') phenotype and tyrosine-phosphorylation of the CagA protein during infection of AGS cells with *H. pylori*.
A. AGS cells were infected with *H. pylori* wild-type P1 or P1Δ*cagA* mutant. Phenotypic changes in *H. pylori* wild-type infected host cells were characterized by spreading and elongation of the cells to form needle-like structures (20–70 μm). Some examples are marked with double arrows. Scale bar = 10 μm.
B. AGS cells were infected with *H. pylori* wild-type P1, P1Δ*virB11*, P1Δ*cagA* and P1Δ*cagA* complemented with intact *cagA* of strain 26695 (P1Δ*cagA/cagA*). The number of cells with typical elongated phenotype was determined in a time course (upper panel). One hundred AGS cells were counted per experiment in triplicate samples. The results are the mean of three independent experiments. The amount of tyrosine-phosphorylated CagA accumulating in AGS cells correlated with the number of cells showing the hummingbird phenotype (lower panel).
C. Tyrosine-phosphorylation of CagA in AGS cells infected with *H. pylori* wild-type P1, *cag* PAI mutants and P1Δ*cagA/cagA*. The upper panel shows a blot that was probed with an antiphosphotyrosine antibody (Anti-p-Tyr) followed by stripping and reprobing with anti-CagA antibody Ab-3 (lower panel). *H. pylori* infection was for 4 h using a MOI of 100. Arrows indicate the position of CagA on the blot. Phosphorylation of CagA always correlated with the expression of the hummingbird phenotype in AGS cells as indicated below (see also Table 1).

Fig. 3. Tyrosine-phosphorylation of CagA occurs in the C-terminus of the protein.
A and B. 2-DE immunoblotting of *H. pylori* without host cell contact (A) and with AGS cell contact (B) identified three CagA protein species (arrowheads): (i) full-length 135 kDa CagA protein ($p135^{CagA}$), (ii) a 100 kDa fragment ($p100^{CagA}$), and (iii) a 35 kDa fragment ($p35^{CagA}$) using anti-CagA antibody Ab-1.
C and D. This blot was stripped and reprobed with anti-CagA antibody Ab-2 (C) and antiphosphotyrosine antibody PY-99 (D) respectively. The antiphosphotyrosine blot (Anti-p-Tyr) revealed a broad signal for phosphorylated $p135^{CagA}$ with a lower pI (6.7–6.8) compared with the non-phosphorylated $p135^{CagA}$ protein form (pI = 6.9).
E and F. As a control, a 2-DE blot of AGS cells infected with P1Δ*cagA* mutant was probed with anti-CagA (E) and antiphosphotyrosine antibodies (F).
G. Schematic presentation of the CagA protein sequence from *H. pylori* strain 26695 (Tomb *et al.*, 1997) and the polypeptides that were used for immunization and production of anti-CagA antibodies Ab-1 and Ab-2 as described in *Experimental procedures*.
Immunoblot analysis with these antibodies in (A–C) specifically detected $p135^{CagA\ and}$ $p100^{CagA}$. In addition, only Ab-1 detected $p35^{CagA}$. This is in agreement with the determination of the pI from full-length and CagA fragments by theoretical analysis and 2-DE suggesting that $p100^{CagA}$ corresponds to the N-terminal part and $p35^{CagA}$ represents the C-terminus of the protein. The exact cleavage/breakage position is unknown. $p35^{CagA}$ strongly reacted with the antiphosphotyrosine antibody, suggesting that tyrosine phosphorylation occurs in the C-terminus of CagA.

CagA in more detail. To localize the tyrosine phosphorylation site on CagA, we first applied two-dimensional gel electrophoresis and immunoblotting experiments with an anti-phosphotyrosine antibody and two anti-CagA antibodies produced against different polypeptides of the protein (Fig. 3). In agreement with our previous study (Moese *et al*., 2001), we detected one predominant spot representing full-length CagA (135 kDa, $p135^{CagA}$) in cultivated *H. pylori* cells and, after longer exposure, two additional spots of 100 kDa ($p100^{CagA}$) and 35 kDa ($p35^{CagA}$) using anti-CagA antibody Ab-1 (Fig. 3A, arrowheads). This suggested that CagA can be processed into



two subfragments in the absence of host cell contact. During infection, the amount of $p100^{CagA}$ and $p35^{CagA}$ steadily increased by four to five times, suggesting that processing is enhanced after translocation of the protein into the host cell (Fig. 3B). To confirm the identity of the CagA protein species, this blot was stripped and reprobed with anti-CagA antibody Ab-2 recognizing the N-terminal part of CagA. Whereas Ab-1 identified each of the three CagA protein spots (Fig. 3B), anti-CagA antibody Ab-2 recognized $p135^{CagA}$ and $p100^{CagA}$ but not $p35^{CagA}$ (Fig. 3C). This finding was in agreement with the theoretical pI values of CagA fragments and proved that $p100^{CagA}$ represented the N-terminus of CagA, and $p35^{CagA}$ corresponded to the remaining C-terminal part (Fig. 3G). The blot shown in Fig. 3B and C was stripped and reprobed with an anti-phosphotyrosine antibody (Fig. 3D). Tyrosine-phosphorylated $p135^{CagA}$ was detected as a broad signal with a more acidic pI (6.7–6.8) compared with the non-phosphorylated form of $p135^{CagA}$ (pI = 6.9). Moreover, both $p100^{CagA}$ and $p35^{CagA}$ reacted with the anti-phosphotyrosine antibody, although with different intensities. The spot detected for $p35^{CagA}$ was about 10 times more intense. This indicated that tyrosine phosphorylation of CagA occurred mainly at the C-terminus of the protein. As a control, infection of AGS cells with P1Δ*cagA* mutant revealed no signals with the anti-CagA antibody (Fig. 3E) and no anti-phosphotyrosine signals in the 100–150 kDa (pI = 6–9) and the 35 kDa (pI = 9–10) sections of the two-dimensional electrophoretic gel (Fig. 3F).

Identification of the CagA tyrosine phosphorylation site in transfected CagA–GFP fusions

To identify the tyrosine phosphorylation site, several fragments of CagA were subcloned as N-terminal fusions to the green fluorescent protein (GFP). These polypeptides included N- and C-terminal sequences of CagA, which also covered two putative tyrosine phosphorylation sites (Backert *et al*., 2000; Odenbreit *et al*., 2000) with homology to consensus motifs of eukaryotic kinases, the Lys–Phe–Gly–Asp–Gln–Arg–Tyr (KFGDQRY) sequence (construct 1) and the Lys–Asn–Ser–Thr–Glu–Pro–Ile–Tyr (KNSTEPIY) sequence (construct 2) (Fig. 4A). CagA construct 1 contained only one tyrosine residue (Y-122), and construct 2 contained three tyrosine residues altogether arranged in three Glu–Pro–Ile–Tyr (EPIY) sequence repeats (Fig. 4A). Sequence alignment of the putative CagA tyrosine phosphorylation sites showed that Y-122 and Y-972 but not Y-899 and Y-918 are well conserved among the *H. pylori* type I strains used in our study (Fig. 4B, Table 1). CagA of strain J99, as an exception, was found to be mutated in most of these motifs and did not become tyrosine phosphorylated during infection (Table 1). Transient expression of the CagA–GFP constructs in AGS cells (Fig. 4C) or HeLa cells (data not shown) identified only one CagA peptide (construct 2) that resulted in tyrosine phosphorylation of the GFP fusion protein. Single phenylalanine substitution of tyrosine residues Y-899, Y-918 and Y-972 in each of the EPIY repeats in construct 2 and subsequent transient expression of the CagA–GFP constructs in AGS cells revealed that $CagA^{Y972F}$ but not $CagA^{Y899F}$ or $CagA^{Y918F}$ affected tyrosine phosphorylation of the GFP fusion protein (Fig. 4C). Collectively, tyrosine Y-972 is conserved in several *H. pylori* CagA variants and is important for phosphorylation of the CagA–GFP fusion, suggesting that this residue might be a CagA phosphorylation site.

Tyrosine residue 972 of CagA is essential for tyrosine phosphorylation and induction of the scattering ('hummingbird') phenotype in AGS cells

We also wanted to analyse the significance of individual CagA tyrosine residues in phosphorylation events and subsequent phenotypic changes of AGS cells during infection with *H. pylori*. For this purpose, Y-122, Y-899, Y-918 and Y-972 were substituted by phenylalanines in CagA expressed from complementation vector pHel2. Membrane and cytoplasmic fractions of AGS cells infected with these *H. pylori* strains were analysed by immunoblot analysis (Fig. 5). Phosphorylated CagA was present in the host cell membrane as well as in the cytoplasm during infection with *H. pylori* wild type, P1Δ*cagA* complemented with CagA wild type, $CagA^{Y122F}$, $CagA^{Y899F}$ and $CagA^{Y918F}$ but not $CagA^{Y972F}$ (Fig. 5, top). Reprobing of the blots with an anti-CagA antibody verified that all CagA derivatives were expressed and successfully translocated into the host cytosol (Fig. 5, top). Thus, the absence of tyrosine-phosphorylated $CagA^{Y972F}$ in the fractions is not the result of impaired translocation. To ensure that the presence of CagA in the host cytoplasm is not a preparation artefact, the blots were reprobed with an antibody against *H. pylori* urease (Fig. 5, bottom), a secreted protein that is highly expressed in the bacteria (Vanet and Labigne, 1998). Fractionation showed the presence of urease exclusively in the membrane fraction because of attached bacteria, thus excluding cross-contamination and suggesting that the presence of CagA proteins in the host cell membrane and cytoplasmic fractions results from translocation of the protein from the bacterium into the host. $CagA^{Y972F}$ prevented the ability of *H. pylori* to induce the hummingbird phenotype in host cells but not $CagA^{Y122F}$, $CagA^{Y899F}$ or $CagA^{Y918F}$ mutants (Fig. 6A). About 60–65% of the AGS cells showed this phenotype during infection with *H. pylori* carrying $CagA^{Y122F}$, $CagA^{Y899F}$ or $CagA^{Y918F}$ mutations, whereas infection with $CagA^{Y972F}$ reduced the number of elongated

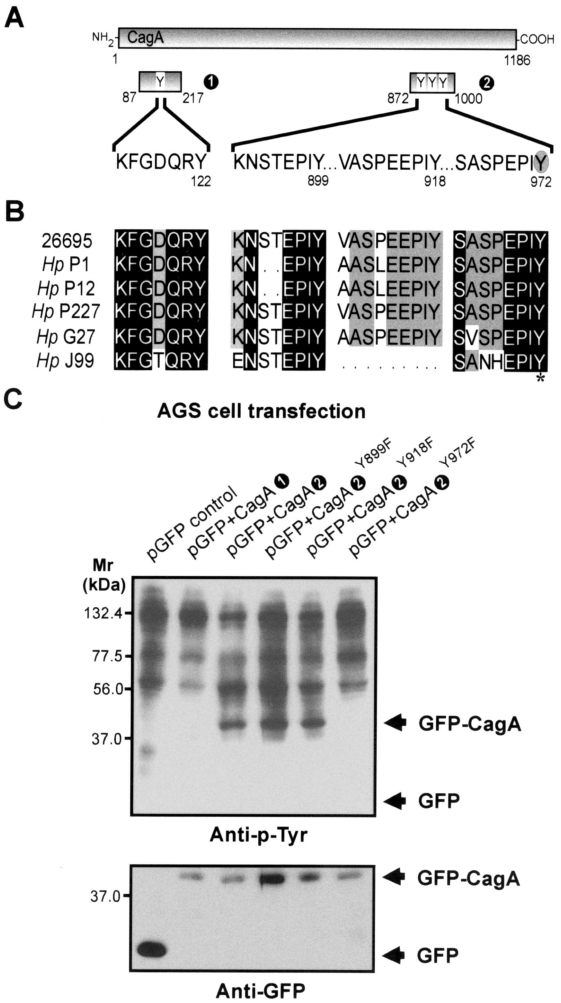


Fig. 4. Detection of a C-terminal CagA fragment that contains a tyrosine-phosphorylation site.
A. Schematic presentation of the CagA protein from *H. pylori* strain 26695 (Tomb *et al*., 1997) and the position of two CagA sequences that were subcloned as N-terminal fusions to the GFP protein. The numbers under the bars represent the first and the last amino acids of CagA. Each construct has a number (black circle). Construct 1 contained one tyrosine residue and construct 2 contained three tyrosine residues. Flanking motif sequences of all tyrosines are indicated (bottom). The numbers under the tyrosines represent the position in the complete CagA protein sequence.
B. Sequence analysis of putative tyrosine phosphorylation motifs in CagA variants used in this study. An alignment of CagA sequences from *H. pylori* strains P1, P12, P227, G27 (sequenced in this study), 26695 (Tomb *et al*., 1997) and J99 (Alm *et al*., 1998) is shown. The motifs show homology to eukaryotic tyrosine phosphorylation consensus sequence [RK]-x(2,3)-[DE]-x(2,3)-Y (Hunter, 1982; Patschinsky *et al*., 1982). At least six identical amino acids are white and boxed with black. At least five identical residues are black and boxed with grey. Phenylalanine substitutions of each of the tyrosines in construct 2 were produced that were used for transfection of host cells.
C. Phosphotyrosine patterns (Anti-p-Tyr) of AGS cell proteins transfected with the constructs shown in (A). Proteins transiently expressed from CagA-GFP construct 2 but not construct 1 were tyrosine-phosphorylated (see arrows). Phenylalanine substitution of Y^{972} abrogated the phosphorylation of construct 2. As control, the blot was stripped and reprobed with anti-GFP antibody (bottom).

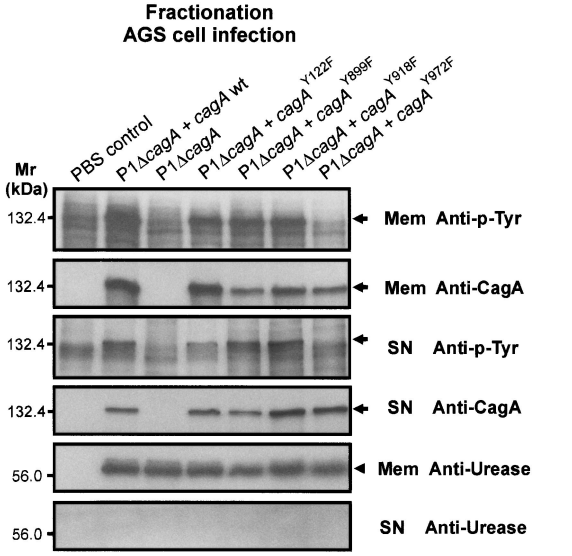


Fig. 5. Determination of the CagA phosphorylation site (Y^{972}) by site-directed mutagenesis of the CagA complementation shuttle vector pHel2 (P1Δ*cagA/cagA*) and cellular fractionation. To identify the *in vivo* tyrosine phosphorylation site in CagA, single phenylalanine substitutions of tyrosine residues in putative phosphorylation motifs were produced in the constructs. Infected AGS cells were fractionated into membrane (Mem) and cytosolic supernatant (SN) fractions that were analysed by immunoblotting using an antiphosphotyrosine antibody (Anti-p-Tyr). The blot was stripped and reprobed with anti-CagA antibody Ab-3 and, as control, with an anti-urease antibody. Arrows indicate the position of CagA on the blot. The arrowhead shows the position of urease.

and spread cells to $<$ 6% (Fig. 6B). This demonstrated that not only translocation but also phosphorylation of CagA at residue Y-972 is important in signalling events leading to the induction of the hummingbird phenotype in AGS cells.

Phosphorylated CagA is essential but not sufficient for induction of the hummingbird phenotype in AGS cells

To determine whether CagA is the only bacterial factor involved in induction of the hummingbird phenotype, CagA was transiently expressed in AGS cells. Transfection of AGS cells with GFP fusion construct 2 (containing phosphorylation site Y-972) or complete CagA did not result in the formation of the hummingbird phenotype after 12, 24 or 48 h (Fig. 7, top). This suggested that the expression of CagA in host cells is not sufficient for the induction of the hummingbird phenotype and, therefore, additional bacterial factor(s) could be involved. To test this hypothesis, transfected AGS cells were infected with *H. pylori* P1Δ*cagA* mutant. Infection with P1Δ*cagA* without prior transfection did not result in induction of the cellular phenotype (Fig. 1). Transfection of AGS cells followed by infection with *H. pylori* P1Δ*cagA* mutant restored the cellular phenotype for full-length CagA but not for construct 2 (Fig. 7, bottom). Here, the hummingbird

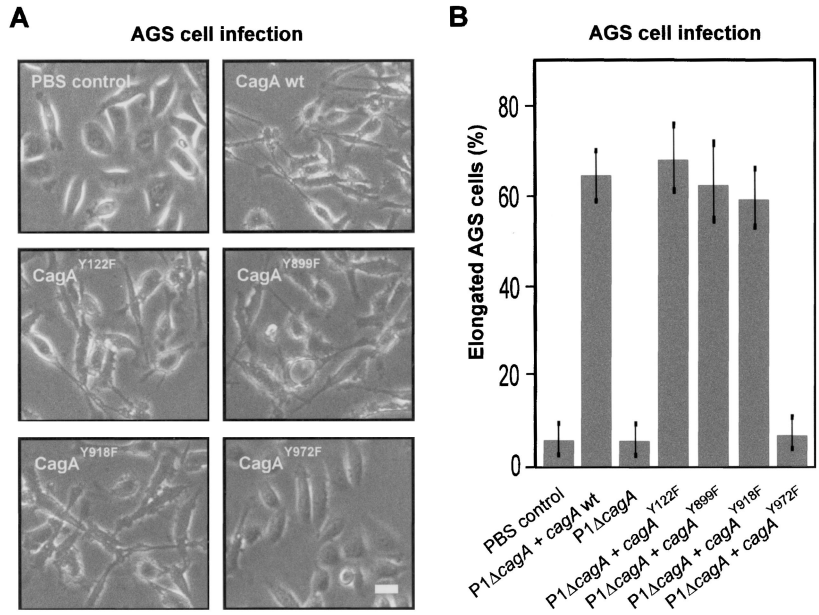


Fig. 6. Translocation and phosphorylation of Y^{972} in the CagA sequence is essential for induction of the scattering ('hummingbird') phenotype in infected AGS cells.
A. Tyrosine residue Y^{972} was found to be essential for both phosphorylation of CagA and induction of the hummingbird phenotype in AGS cells as analysed by phase contrast microscopy. In each experiment *H. pylori* infection was synchronized using a MOI of 100 and continued for 4 h. Scale bar = 10 μm.
B. Quantitative evaluation of AGS cells with typical elongated phenotype after 4 h of infection. One hundred AGS cells were counted per experiment in triplicate samples. The results are the mean of three independent experiments.

phenotype was detected in at least 50% of the transfected cells that were infected with P1Δ*cagA.* These results collectively suggested that: (i) the CagA protein plays a crucial role; (ii) the C-terminus of CagA containing the phosphorylation site Y-972 is important but not sufficient; and (iii) full-length CagA alone is not sufficient and needs at least one more bacterial factor to induce signalling events leading to the induction of the hummingbird phenotype in infected host cells.

Tyrosine phosphorylation of CagA at Y-972 is necessary for cytoskeletal rearrangements in infected AGS cells

Confocal microscopy was used to characterize CagA-dependent actin cytoskeletal rearrangements and the hummingbird phenotype. For this purpose, AGS cells were infected with P1Δ*cagA* complemented with intact CagA or $CagA^{Y972F}$ mutant and stained for CagA, tyrosine-phosphorylated proteins as well as for F-actin. The projection of *z* stack *xy* images shown represents entire infected cells (Fig. 8). As expected, P1Δ*cagA/cagA* induced elongation of the infected cells (Fig. 8, top), which were not observed for *H. pylori* expressing CagA with a tyrosine to phenylalanine substitution at Y-972 (Fig. 8, bottom left). These phenotypic changes in the host cell were characterized by spreading and elongation of the cellular matrix, resulting in a strong polarization of the cells to form needle-like structures full of F-actin. This indicated the occurrence of drastic actin cytoskeletal rearrangements in infected AGS cells promoted by phosphorylated wild-type CagA. Dephosphorylation of proteins in the focal adhesions, as known from *Yersinia* YopH protein (e.g.

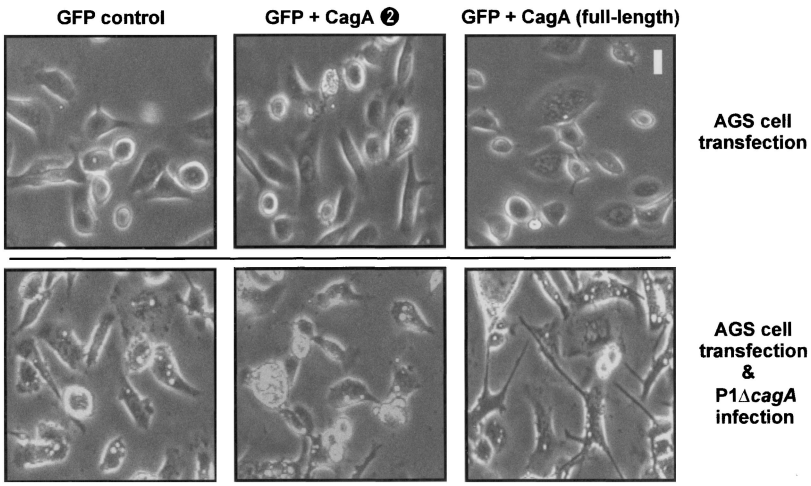


Fig. 7. Phosphorylated CagA is essential but not sufficient for induction of the scattering ('hummingbird') phenotype in AGS cells. Phase contrast micrographs of AGS cells transfected with CagA-GFP fusion constructs (upper panel). Twelve hours after transfection, AGS cells were infected with P1Δ*cagA* mutant using a MOI of 100. Only the full-length CagA construct induced the hummingbird phenotype in at least 50% of the transfected cells after infection with P1Δ*cagA* (lower panel). Scale bar = 10 μm.

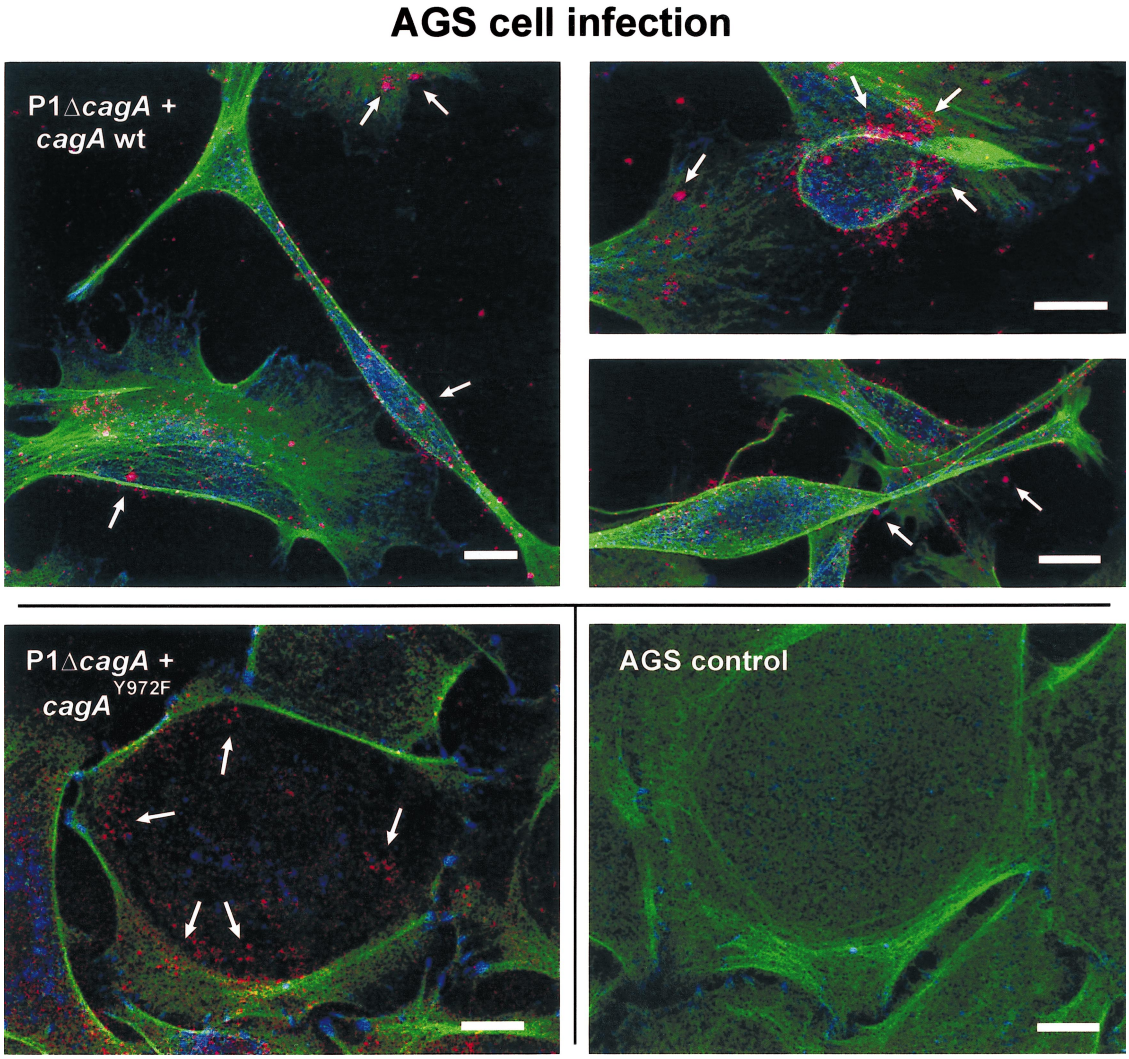


Fig. 8. Confocal laser scanning microscopy of AGS cells infected with *H. pylori* P1Δ*cagA/cag* wild-type (upper panels) or P1Δ*cagA/cagA*^{Y972F} (lower panel, left). The images are the projection of *z* stack *xy* images and show entire cells. *H. pylori* P1Δ*cagA/cagA* but not P1Δ*cagA/cagA*^{Y972F} induced elongation and spreading of the cells that were typically observed for the hummingbird phenotype. CagA is shown in red, actin is shown in green and phosphorylated proteins are labelled with blue. Areas of co-localization of CagA and phosphotyrosine appear purple. Examples of translocated CagA signals are marked with arrows. As control, uninfected AGS cells showed no cross-contamination with anti-CagA signals (lower panel, right). Scale bars = 10 μm.

Black and Bliska, 1997; Cornelis and Wolf-Watz, 1997), was not detected during *H. pylori* infection, and phosphorylated CagA did not co-localize with focal adhesions. Instead, phosphorylated CagA was distributed in the host cytoplasm and, more often, in the host membrane directly beneath the attached bacteria. Control, uninfected AGS cells did not reveal anti-CagA signals (Fig. 8, bottom right). Examples of CagA co-localizing with anti-phosphotyrosine signals (purple colour) during infection with P1Δ*cagA/cagA* are indicated by arrows (Fig. 8, top). These signals were carefully checked by the analysis of single optical sections. In agreement with the biochemical fractionation studies described above, CagA^{Y972F} was detected in the host membrane and cytosol, showing that CagA was successfully translocated from the bacterium into the host cell (Fig. 8, bottom, see arrows). However, no co-localization of CagA^{Y972F} with signals of the anti-phosphotyrosine staining was detected. Infection with this mutant also did not significantly change phosphotyrosine or F-actin signals within the host cell. Thus, unlike the CagA^{Y122F}, CagA^{Y899F} and CagA^{Y918F} mutants that did not affect CagA tyrosine phosphorylation and infection-induced actin rearrangements (like wild-type CagA), CagA carrying the Y972F substitution was not phosphorylated, and AGS cells infected with this mutant failed to rearrange actin filaments.

Discussion

The correlation between the expression of CagA and *H.*

pylori virulence was described a long time ago (Covacci *et al*., 1993; Tummuru *et al*., 1993), but the function of this protein is still not understood. Recent investigations have demonstrated type IV secretion system-mediated delivery of CagA into host cells followed by tyrosine phosphorylation and have provided initial evidence for the role of this protein during the infection process (Segal *et al*., 1999; Asahi *et al*., 2000; Backert *et al*., 2000; Odenbreit *et al*., 2000; Stein *et al*., 2000). In this report, we provide clear genetic evidence for the function of CagA and CagA phosphorylation in host signalling events that result in rearrangements of the actin cytoskeleton. We identified one tyrosine phosphorylation site of CagA by site-directed mutagenesis. Several lines of evidence demonstrated that CagA is phosphorylated on residue Y-972, which is crucial for the induction of the scattering ('hummingbird') phenotype in gastric epithelial cells (AGS). These novel findings may lead to a better understanding of the function of the *H. pylori* type CagA protein and its role in bacterial pathogenesis.

To identify the CagA tyrosine phosphorylation site, we have generated several CagA wild-type and mutant constructs. Site-directed mutagenesis of both (i) transiently expressed CagA–GFP fusion proteins and (ii) constructs expressing CagA in *H. pylori* has lead to the identification of the tyrosine phosphorylation site (Y-972) of the protein. This finding showed that the two previously proposed phosphorylation motifs at Y-122 and Y-899 (Backert *et al*., 2000; Odenbreit *et al*., 2000) were not essential for CagA phosphorylation. Sequence comparison showed that Y-972 is well conserved among five CagA variants used in this study and many other CagA sequences (our unpublished data) available in databases. Although we cannot exclude the possibility that other tyrosine phosphorylation sites are present in other CagA variants, it is reasonable to assume that the SASPEPIY sequence motif and particularly Y-972 is of general importance for CagA phosphorylation. *In vitro*, c-src and epidermal growth factor receptor protein kinases have been shown to phosphorylate CagA (Asahi *et al*., 2000). Phosphorylation of CagA was usually detected during infection of gastric epithelial cells, but the *in vivo* kinase is unknown. Interestingly, phosphorylation of a C-terminal CagA–GFP fusion protein was detected in both AGS and HeLa cell transfections, which led us to suggest that CagA is phosphorylated by a ubiquitously expressed eukaryotic tyrosine kinase active in these cell types.

A variety of biochemical fractionation and microscopic studies has clearly shown that translocation and phosphorylation of CagA are essential to induce cytoskeletal changes in the host. CagA-dependent phenotypic changes in AGS cells were time and dose dependent and were also seen in the non-gastric epithelial cell line Me180 (our unpublished data). This supports our view that induction of this type of cell transformation is a common theme of the *H. pylori* infection and is not restricted to only one cell type. The scattering (or 'hummingbird') phenotype of AGS cells is characterized by the dramatic elongation and spreading of host cells and was first observed by Segal *et al*. (1999). The location of CagA was often associated with regions of active actin reorganization such as microspike formation and ruffles. This is reminiscent of the findings that: (i) several other bacterial toxins interfere with growth factor signalling pathways (cf. Popoff, 1998); and (ii) phenotypic changes in MDCK cells were induced by the activation of the c-Met receptor by hepatocyte growth factor HGF (cf. Ridley *et al*., 1995). Here, we have clearly demonstrated that the hummingbird phenotype in AGS cells is induced by *H. pylori* type I strains but not by type II strains nor by isogenic *vir* gene mutants, and that the induction of this phenotype depended on phosphorylation of CagA residue Y-972. Transient expression of CagA in AGS cells did not lead to the induction of cytoskeletal rearrangements; however, infection of the transfected cells with P1Δ*cagA* mutant resulted in the restoration of the hummingbird phenotype. This supported the idea that CagA plays a key role but might not be the only bacterial factor involved in *H. pylori*-induced cytoskeletal rearrangements. It seems likely that phosphorylated CagA needs at least one additional factor. The vacuolating toxin of *H. pylori* (VacA), which targets the mitochondria (Galmiche *et al*., 2000) and interferes with vesicle trafficking by causing the accumulation of endosomal–lysosomal vacuoles containing Rab7 (Papini *et al*., 1997), does not play a role in either of the described host response phenotypes (Segal *et al*., 1999; this study). Thus, attachment of *H. pylori* to the host cell and subsequent *cag* PAI-independent signalling events (e.g. Segal *et al*., 1999; Wessler *et al*., 2000), bacterial lipopolysaccharide (LPS)-induced signalling (Kawahara *et al*., 2001) or translocation of other virulence factor(s) by the type IV system contribute to the CagA-induced phenotypic response. For example, Rho GTPases Rac1 and Cdc42 are activated in a *cag* PAI-dependent but CagA-independent manner and recruited to the sites of bacterial attachment (Churin *et al*., 2001). Therefore, the activation of Rac1 and Cdc42 (by an as yet unknown bacterial factor) might contribute to the CagA phosphorylation-dependent hummingbird phenotype in AGS cells.

This study proves that phosphorylation of CagA at Y-972 has a function in actin-based cytoskeletal rearrangements; however, interacting partners of CagA in the host cell have not yet been identified. Database searches of CagA sequences did not reveal significant homologies to any known proteins. Our sequence analysis revealed that CagA contains a SH3 domain binding motif (our unpublished data). This suggests that CagA might interact with a SH3 domain containing host adapter

molecule to induce host signalling involved in the induction of the hummingbird phenotype. However, once translocated and tyrosine phosphorylated, CagA is also likely to be recognized by an SH2 domain-containing host protein. This $CagA^{P\text{-}Tyr}$–SH2 protein complex could stimulate multiple signalling cascades involved in the reorganization of the actin cytoskeleton, possibly involving the N-WASP and Arp2/3 complex as suggested recently (Censini *et al*., 2001). Whether processing of full-length CagA ($p135^{CagA}$) into N-terminal $p100^{CagA}$ and C-terminal $p35^{CagA}$ fragments plays a role in these processes is at present unknown. Previous studies reported that translocation and tyrosine phosphorylation of CagA are temporally correlated with dephosphorylation of as yet unidentified host proteins p80 and p120 (Backert *et al*., 2000; Odenbreit *et al*., 2000). Thus, a direct correlation or even interaction of phosphorylated CagA with p80 and/or p120 host proteins can be suggested. Our confocal data showed that proteins in the focal adhesion complex were not dephosphorylated and, therefore, the *H. pylori* infection is different from that of *Yersinia*, in which YopH acts as a PTPase with $p130^{Cas}$ and $p115^{FAK}$ as possible targets (e.g. Black and Bliska, 1997; Cornelis and Wolf-Watz, 1997). Whether translocated CagA itself expresses PTPase activity or recruits a host cell PTPase is still unclear.

Various pathogens trigger actin polymerization by delivering effector proteins into the host cell. For example, ActA (*Listeria monocytogenes*) and IcsA (*Shigella flexneri*) mimic or stimulate N-WASP respectively (Welch *et al*., 1998; Egile *et al*., 1999; Loisel *et al*., 1999). The characteristics of *H. pylori* infection have led several investigators to speculate about an analogy with the intimin receptor Tir of enteropathogenic *Escherichia coli* (EPEC) (Kenny *et al*., 1997). Interestingly, there is indeed an analogy, because Tir and CagA are translocated and undergo tyrosine phosphorylation at the C-terminus, CagA at Y-972 (this study) and Tir at Y-474 (Kenny, 1999). Site-directed mutagenesis demonstrated that phosphorylation of Tir at Y-474 is essential for actin nucleation activity and pedestal formation (Kenny, 1999), whereas the N-terminus of Tir interacts directly with host cytoskeletal proteins (Freeman *et al*., 2000; Goosney *et al*., 2000). CagA and Tir have no sequence homology throughout their molecules. Tir contains two transmembrane domains (Luo *et al*., 2000). In contrast, CagA has no such domains, is a highly hydrophilic protein (Covacci *et al*., 1993) and might form cylinder structures (Segal *et al*., 1999). However, fractionation and confocal microscopic studies have shown that CagA mainly associates with the host membrane. Tir anchors the bacterium directly to the host cytoskeleton via α-actinin binding at the N-terminus (Freeman *et al*., 2000) and acts as a dimeric receptor for intimin-dependent bacterial attachment. *H. pylori* mutants lacking CagA do not show significantly lower binding capacity to AGS cells compared with wild-type *H. pylori* (our unpublished data), suggesting that CagA is not a receptor but has a different function.

In conclusion, using a combination of different molecular approaches, we have demonstrated the dramatic consequences of CagA translocation and Y-972 phosphorylation in the host cell. The phenotypical changes in infected AGS cells are associated with CagA size modification and the activation of host signalling events, which may be crucial for *H. pylori* virulence. To unravel hidden features of *H. pylori* host interactions, future work should address the identification of other translocated bacterial factors and the characterization of host cell molecules interacting with CagA.

Experimental procedures

Helicobacter pylori *strains and the production of* cag *PAI mutants*

Helicobacter pylori strains P1, P12, P227, 1061 and G27 are clinical isolates that have been described previously (Censini *et al*., 1996; Gomez-Duarte *et al*., 1998; Heuermann and Haas, 1998; Backert *et al*., 2000). *H. pylori* strains 26695 *(*Tomb *et al*., 1997*) and* J99 (Alm *et al*., 1999) were chosen because their genomes have been entirely sequenced. Isogenic Δ*cagA and* Δ*vir* gene knock-out mutants in strain P1 (Table 1) have been constructed by insertion of a chloramphenicol resistance gene cassette (Cm^R, 1 kb *Bam*HI/*Bgl*II fragment of plasmid pTnMax1) according to a standard protocol (Heuermann and Haas, 1998; *Backert et al*., 2000). The construction, position and orientation of the Cm^R cassette in each of these mutants was described in detail (Moese *et al*., 2001). All *H. pylori* strains were grown on horse serum agar plates supplemented with vancomycin ($10\,\mu g\,ml^{-1}$), nystatin ($1\,\mu g\,ml^{-1}$) and trimethoprim ($5\,\mu g\,ml^{-1}$), and, if necessary, with chloramphenicol (4–$6\,\mu g\,ml^{-1}$) and/or kanamycin ($20\,\mu g\,ml^{-1}$). Incubation was performed at 37°C for 2 d in an anaerobic jar containing a campygen gas mix of 5% O_2, 10% CO_2 and 85% N_2 (Oxoid).

Cloning of cagA *genes, sequence analysis and* cagA *complementation*

To analyse the conservation of putative tyrosine phosphorylation motifs in different CagA proteins, *cagA* subfragments from *H. pylori* strains P1, P12, P227 and G27 were amplified by polymerase chain reaction (PCR) using the following primers. Forward primer: 5′-CATCTTTAGCGTTGCATT TGATTT, reverse primer: 5′-CAACACAAGTAGCCCCTA AAACTT. All PCR products were cloned into the pCR2.1 vector (Invitrogen) and sequenced. The HUSAR program (version 4.0, German Cancer Research Centre; http://www.genius.embnet.dkfz-heidelberg.de) was used for *cagA* sequence analysis. The pI from different CagA fragments was calculated using EXPASY PROTEOMICS tools (http://www.expasy.ch/tools/). For construction of a complementation vector, *cagA* containing its own promoter was amplified from

strain 26695 (NCBI database accession number: AAD07614) using primers 5′-GTCGACCATCTTTAGCGTTGCATTTGATTT (forward) and 5′-GTCGACCAACACAAGTAGCCCCTAAAACTT (reverse). Both primers contained unique *Acc*I sites that were used to clone *cagA* in the *Cla*I site of the *E. coli/H. pylori* shuttle vector pHel2 containing the *oriT* of RP4 and a kanamycin resistance gene cassette (*aph-A3*) as a selectable marker (Heuermann and Haas, 1998). Site-directed mutagenesis of tyrosine residues 122, 899, 918 and 972 in the CagA sequence was done using the Sculptor *in vitro* mutagenesis kit according to the instructions of the supplier (Amersham Pharmacia Biotech). All vectors were transferred into *H. pylori* by conjugation of *E. coli* XL1-blue with P1*cagA* using *E. coli* GC7[pRK2013] as mobilizer (Heuermann and Haas, 1998). To confirm that recombination of vectors with the *H. pylori* chromosome has not taken place, plasmids were reisolated and RFLP patterns were confirmed. CagA phosphorylation motifs were sequenced.

Synchronized infection assays

AGS cells (ATCC CRL 1739; a human gastric adenocarcinoma epithelial cell line) were grown in 25 cm^2 tissue culture flasks containing RPMI-1640 medium (Gibco BRL) complemented with 10% fetal bovine serum (FBS, Gibco BRL) for 2 d to reach monolayers of about 70% cell confluency. The cells were washed once with phosphate-buffered saline (PBS) and 4 ml of fresh medium was added to each flask. *H. pylori* (2×10^8) were suspended in 0.5 ml of PBS and added to 2×10^6 AGS cells at a multiplicity of infection (MOI) of 100. Infection was synchronized by centrifugation for 5 min at 600 *g*. For kinase inhibition studies, the cell culture medium was complemented with 250 μM genistein before and during infection. After incubation in a 5% CO_2/95% air incubator for 2–4 h, infected AGS cells were washed once with PBS (containing 1 mM Na_3VO_4; Sigma-Aldrich) to remove non-adherent *H. pylori*. Whole cell lysates with attached bacteria were made by pelleting the cells at 600 *g* and 4°C. The cell pellets were washed again with precooled PBS and sonicated in the presence of proteinase and phosphatase inhibitors (1 mM PMSF, 2 μg ml^{-1} aprotinin, 2 μg ml^{-1} leupeptin, 1 mM Na_3VO_4). Resulting protein lysates were suspended in 1 × SDS–polyacrylamide gel electrophoresis (PAGE) buffer (100 mM Tris-HCl pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromphenolblue, 20% glycerol).

Construction of CagA-GFP fusion constructs and transfection assay

Full-length *cagA* and two 400 bp *cagA* subfragments of *H. pylori* strain 26695 were amplified by PCR with primers containing unique *Sal*I and *Sgr*AI/*Age*I sites respectively. Full-length CagA: 5′-GTCGACCACCATGACTAACGAAACTATTGATCAAACAAG (forward), 5′-CACCGGTGGAGATTTTTGGAAACCACCTTTTG (reverse); construct 1: 5′-GTCGACCACCATGTATTTTTCAGACTTTATCGATAAG (forward), 5′-ACCGGTAGGAGAAAAATATCCAACCAATCCCC (reverse); construct 2: 5′-GTCGACCACCATGTCGGATATCAAGAAAGAATTGAATG (forward), 5′-ACCGGTAGCCTTGATCGCCCTACCTTAC (reverse). The latter two PCR products contained a new start codon and were cloned in frame into the *Sal*I and *Age*I sites of the eukaryotic expression vector pEGFP-N1 (Clontech) as N-terminal fusions to the green fluorescence protein (GFP). All plasmid constructs (1 μg) were transfected into 0.8×10^6 AGS or HeLa cells (ATCC CCL-2, a human cervix adenocarcinoma cell line) on six-well plates using the Effectene kit (Qiagen). The data presented are representative of more than five independent experiments.

Cellular fractionation

For the preparation of membrane/nuclear and cytoplasmic fractions of infected host cells, 2×10^6 AGS cells were washed with precooled PBS (containing 1 mM Na_3VO_4) to remove non-adherent bacteria. The cells were harvested by centrifugation at 600 *g* for 5 min at 4°C and the pellets were washed again in 1 ml buffer A (10 mM Tris-HCl pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 10% glycerol, 10 mM K_2HPO_4, 1 mM Na_3VO_4, 10 mM NaF, 0.5 mM dithiothreitol (DTT) and 0.5 mM PMSF) followed by centrifugation. The resulting AGS cell pellet was resuspended in 200 μl of buffer A containing 1% nonidet-P40, gently mixed, and incubated for 1 min on ice to release the soluble cytoplasmic fraction in the presence of protease and phosphatase inhibitors. Membrane/nuclear fractions were collected by pelleting at 1000 *g* for 10 min. Cytoplasmic fractions were purified from remaining cell debris by centrifugation at 12 000 *g* for 30 min at 4°C. The fractions were resuspended in an equal amount of 1 × SDS–PAGE buffer.

Immunoblot analysis

Cells were harvested in precooled PBS/1 mM Na_3VO_4 and resuspended in 1 × SDS–PAGE buffer. A detailed protocol for 2-DE was described elsewhere (Jungblut *et al*., 2000; Backert *et al*., 2001). Blotting of proteins was performed onto PVDF membranes (Immobilon-P, Millipore). Phosphorylated and non-phosphorylated CagA proteins were detected by incubation of the membranes with a mouse monoclonal antiphosphotyrosine antibody PY99 (Santa Cruz Biotechnology), a rabbit polyclonal anti-CagA antibody (Ab-1) (Austral Biologicals) produced against CagA residues 748–1015 (Covacci *et al*., 1993). Two additional polyclonal anti-CagA antibodies (Ab-2, Ab-3) were produced against other CagA polypeptides. Ab-2 was produced by immunization of mice with a CagA polypeptide (residues 110–769) derived from a sequence that was reported recently (Tummuru *et al*., 1993). Ab-3, a polyclonal rabbit anti-CagA antibody recognising full-length CagA, $p100^{CagA}$ and $p35^{CagA}$ was a gift of S. Censini and A. Covacci (ISIC) (Covacci *et al*., 1993). Mouse polyclonal antiurease antibodies were described recently (Gomez-Duarte *et al*., 1998). Rabbit anti-GFP polyclonal antibody was purchased from MoBiTec. All antibodies were diluted 1:1000 in TTBS buffer (14 mM NaCl, 25 mM Tris-HCl, pH 7.4; 0.01% Tween 20). Before addition of the antibodies, membranes were blocked with TTBS buffer + 3% bovine serum albumin (BSA) for 1 h at room temperature. As a secondary antibody, horseradish peroxidase-conjugated antimouse or antirabbit polyvalent sheep immunoglobulins (Amersham Pharmacia Biotech) were used in a dilution of 1:5000. Antibody detection was performed with the Renaissance

Western Blot kit system for ECL immunostaining (ICN Biochemicals).

Confocal laser scanning microscopy

Lab-Tek chamber slides (NUNC) or six-well plates containing infected cultures were washed five times with PBS containing 1 mM Na_3VO_4, fixed in 3.8% paraformaldehyde (PFA) for 15 min, washed with PBS and then permeabilized with 0.2% Triton X-100 for 10 min. Samples were washed twice with PBS and processed for immunofluorescence staining. All antibodies were diluted in PBS/0.2% BSA. Filamentous actin in the host cell was labelled with Alexa 488-conjugated phalloidin diluted 1:100 (MoBiTec). Tyrosine-phosphorylated proteins were detected using the monoclonal antibody PY99 and Ab-1 was used to stain for CagA proteins. Both antibodies were used in a 1:1000 dilution. All samples were analysed using a Leica TCS SP confocal laser scanning microscope equipped with an argon/krypton mixed gas laser source (Leica Lasertechnik).

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