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Translocation of the *Helicobacter pylori* CagA protein in gastric epithelial cells by a type IV secretion apparatus

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Summary

***Helicobacter pylori* is one of the most common bacterial pathogens, infecting about 50% of the world population. The presence of a pathogenicity island (PAI) in *H. pylori* has been associated with gastric disease. We present evidence that the *H. pylori* protein encoded by the cytotoxin-associated gene A (*cagA*) is translocated and phosphorylated in infected epithelial cells. Two-dimensional gel electrophoresis (2-DE) of proteins isolated from infected AGS cells revealed *H. pylori* strain-specific and time-dependent tyrosine phosphorylation and dephosphorylation of several 125–135 kDa and 75–80 kDa proteins. Immunoblotting studies, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), cell fractionation and confocal microscopy demonstrated that one of the 125–135 kDa proteins represents the *H. pylori* CagA protein, which is translocated into the host cell membrane and the cytoplasm. Translocation of CagA was dependent on functional *cagA* gene and virulence (*vir*) genes of a type IV secretion apparatus composed of *virB4*, *virB7*, *virB10*, *virB11* and *virD4* encoded in the *cag* PAI of *H. pylori*. Our findings support the view that *H. pylori* actively translocates virulence determinants, including CagA, which could be involved in the development of a variety of gastric disease.**

Introduction

Helicobacter pylori, a Gram-negative microaerophilic bacterial pathogen and class I carcinogen, is specialized to live for decades in the extreme environment of the human stomach. *H. pylori* colonizes and interacts with gastric epithelial cells throughout the host's life and induces gastric inflammation, which can progress to a variety of diseases, such as peptic ulcer, mucosa-associated lymphoma or gastric cancer (Covacci *et al*., 1999; Cover and Blaser, 1999). The recent determination of two complete *H. pylori* genome sequences of strains 26695 and J99 provided hints to the presence of several putative virulence factors (Tomb *et al*., 1997; Alm *et al*., 1999; Taylor, 1999), those studied in most detail being involved in the production of abundant amounts of urease, the expression of the vacuolating toxin (VacA) or CagA and adhesion to tissue-specific cell receptors (Guruge *et al*., 1998; Covacci *et al*., 1999). The major disease-associated genetic difference in *H. pylori* isolates is the presence (*cag*$^+$ or type I strains) or absence (*cag*$^-$ or type II strains) of the *cag* pathogenicity island (PAI), a locus of about 40 kb containing up to 31 genes (Censini *et al*., 1996; Akopyants *et al*., 1998).

Six of the *H. pylori cag* genes are homologous to the well-known virulence genes *virB4*, *virB7*, *virB9*, *virB10*, *virB11* and *virD4* from *Agrobacterium tumefaciens*, *Bordetella pertussis* or *Legionella pneumophila* (Winans *et al*., 1996; Christie, 1997; Covacci *et al*., 1999). By analogy of the *cag* genes to the VirB system of the agrobacterial transfer (T)-DNA-transfer machinery to plant cells, it was suggested recently that the *cag* PAI might code for an ancient type IV secretion transporter that is capable of exporting a variety of proteinaceous material, and probably also nucleoprotein particles (Censini *et al*., 1996; Christie, 1997; Backert *et al*., 1998). In *A. tumefaciens*, VirB4 exhibits ATPase activity and is tightly associated with the inner cytoplasmic membrane. *H. pylori* strain 26695 contains altogether four *virB4* gene copies. One of them is located inside, but three are outside of the *cag* PAI. VirB7 is a lipoprotein that, after recruiting VirB9 to form a heterodimer, is postulated to play a role in stabilizing other agrobacterial VirB proteins by the formation of intermolecular disulphide bridges, leading to the assembly of a functional transporter. This heterodimer seems to stabilize VirB10 in higher order complexes. VirB11 is also essential in the assembly of the transporter and exhibits ATPase activity. A ring-shaped structure composed of six monomeric *H. pylori* VirB11 proteins has been observed *in vitro* (Krause *et al*., 2000). A potential *virB2* gene encoding a pilus-like structure has not been identified yet in *H. pylori*.

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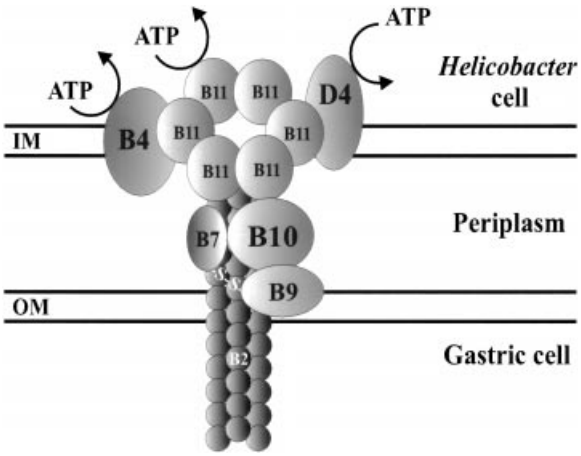

Fig. 1. Hypothetical model of the *H. pylori* type IV secretion machinery encoded in the *cag* PAI. The putative position of the core proteins VirB4, VirB7, VirB9, VirB10, VirB11 and VirD4 in the bacterial inner membrane (IM) and outer membrane (OM) is presented by homology with models of the agrobacterial and *B. pertussis* type IV secretion apparatus (Winans *et al*., 1996; Christie, 1997). The agrobacterial VirB7 and VirB9 proteins form intermolecular heterodimers linked via a disulphide bridge (B7-s-s-B9). Purified *H. pylori* VirB11 proteins form a hexameric ring *in vitro* (Krause *et al*., 2000). Potential ATP-binding domains in the VirB4, VirB11 and VirD4 proteins are indicated as determined from the Prosite database using the HUSAR program (http://www.genius.embnet.dkfz-heidelberg.de). According to this working model, we have produced isogenic knock-out mutants of the respective genes in *H. pylori* strain P1 to investigate their relevance in the *H. pylori* infection process.

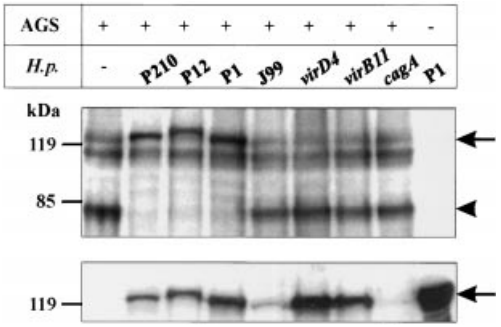

Fig. 2. Phosphorylation of the *H. pylori* CagA protein during infection. Immunoblot analysis of AGS cells infected with *H. pylori* wild-type strains and mutants. Total protein isolated from *H. pylori* strain P1 served as a control. The blot was probed with an antiphosphotyrosine antibody (top) and reprobed with an anti-CagA serum (bottom). Representative results from eight independent experiments are shown. In each experiment, *H. pylori* infection was for 2 h using a MOI of 50. Molecular size markers (in kDa) are indicated.

Interestingly, members of the VirD4 protein family that have been suggested to link the T-DNA complex directly to the exporting membrane channel have only been detected in agrobacterial Ti-plasmid and conjugative DNA transfer systems of broad-host-range plasmids but not in protein transporters, such as that of *B. pertussis* (Pansegrau and Lanka, 1996). However, despite the significance of potential virulence genes present, their functional importance as a protein and/or DNA transporter is not known, and the mechanisms by which *H. pylori* causes gastritis, ulcers or cancer remain poorly understood.

It has been demonstrated that *H. pylori* triggers, in a *cag*-dependent manner, cellular events such as the reorganization of the actin cytoskeleton and pedestal formation (Segal *et al*., 1996), the release of cytokines and the activation of host signalling pathways, leading to the activation of the transcription factors AP-1 and NF-κB (Tummuru *et al*., 1995; Aihara *et al*., 1997; Keates *et al*., 1997; Münzenmaier *et al*., 1997; Sharma *et al*., 1998; Li *et al*., 1999; Naumann *et al*., 1999). The development of severe gastric disease, which is typically associated with an infection of strains carrying the *cag* PAI, suggests that the activation of the above-mentioned components of the proinflammatory cellular response is crucial for the clinical outcome. Although recent data described in detail the cellular host response leading to the transcriptional activation of certain target genes (Naumann *et al*., 1999), no bacterial components directing this signalling have been identified so far. *H. pylori* was recently shown to induce tyrosine phosphorylation of 105–145 kDa proteins (Segal *et al*., 1996; 1997; Su *et al*., 1999), and deconvolution immune fluorescence microscopy suggested that a percentage of bacteria attached to the cell surface is associated with a high concentration of phosphorylated CagA, which could induce cellular changes, such as elongation and spreading of host cells, including the production of filopodia and lamellipodia (Segal *et al*., 1999).

We are interested in clarifying the role of the type IV secretion apparatus of *H. pylori* in the induction of host signalling responses. Our experimental approach involves the identification of bacterial proteins targeted into the host cell. Data presented here demonstrate that CagA is translocated into the host membrane and into the cytoplasm by the *H. pylori* type IV secretion machinery encoded in the *cag* PAI. The CagA protein becomes phosphorylated during infection in a strain- and time-dependent manner. This process is embedded in a scenario of certain protein tyrosine phosphorylation and dephosphorylation events within the host cell that may be crucial for *H. pylori* virulence.

Results

Type IV secretion-dependent phosphorylation of the H. pylori *CagA protein during infection*

In order to understand the molecular mechanisms of the *H. pylori* infection and the potential role of the type IV secretion apparatus encoded in the *cag* PAI, we have

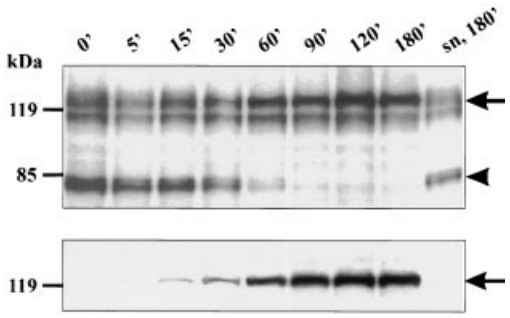

Fig. 3. Time-dependent induction of tyrosine phosphorylation of the CagA protein (see arrow) and dephosphorylation of 75–80 kDa proteins (see arrowhead) in AGS cells upon infection with *H. pylori* wild-type strain P1. As a control, the supernatant (sn) of *H. pylori* strain P1 was added to AGS cells for 180 min. The blot was probed with an antiphosphotyrosine antibody (top) and reprobed with an anti-CagA serum (bottom). Representative results from three independent experiments are shown. A MOI of 50 was used in each experiment. Molecular size markers (in kDa) are indicated.

produced a set of *vir* gene knock-out mutants according to our current working model depicting the putative position of the *H. pylori* Vir proteins assembled as a transporter complex in the bacterial membrane by analogy with the agrobacterial T-DNA secretion machinery (Fig. 1). To study the cellular cross-talk between the pathogen and the host cell, subconfluent monolayers of the human gastric adenocarcinoma cell line AGS were infected with different *H. pylori* strains, followed by analysis of the phosphotyrosine-modified proteins using an anti-phosphotyrosine antibody. Several *H. pylori* type I-wild-type strains, including P1, P12 and P210, induced tyrosine phosphorylation as well as dephosphorylation of 125–135 kDa proteins and dephosphorylation of 75–80 kDa proteins (Fig. 2). Tyrosine phosphorylation and dephosphorylation events have not been detected in the type I strain J99, in the type II strain 1061 lacking the complete *cag* PAI and in mutants for the core components *virB4*, *virB7*, *virB10* (data not shown), *virB11* and *virD4* (Fig. 2, top) of the type IV transporter encoded in the *cag* PAI. These results were obtained using a multiplicity of

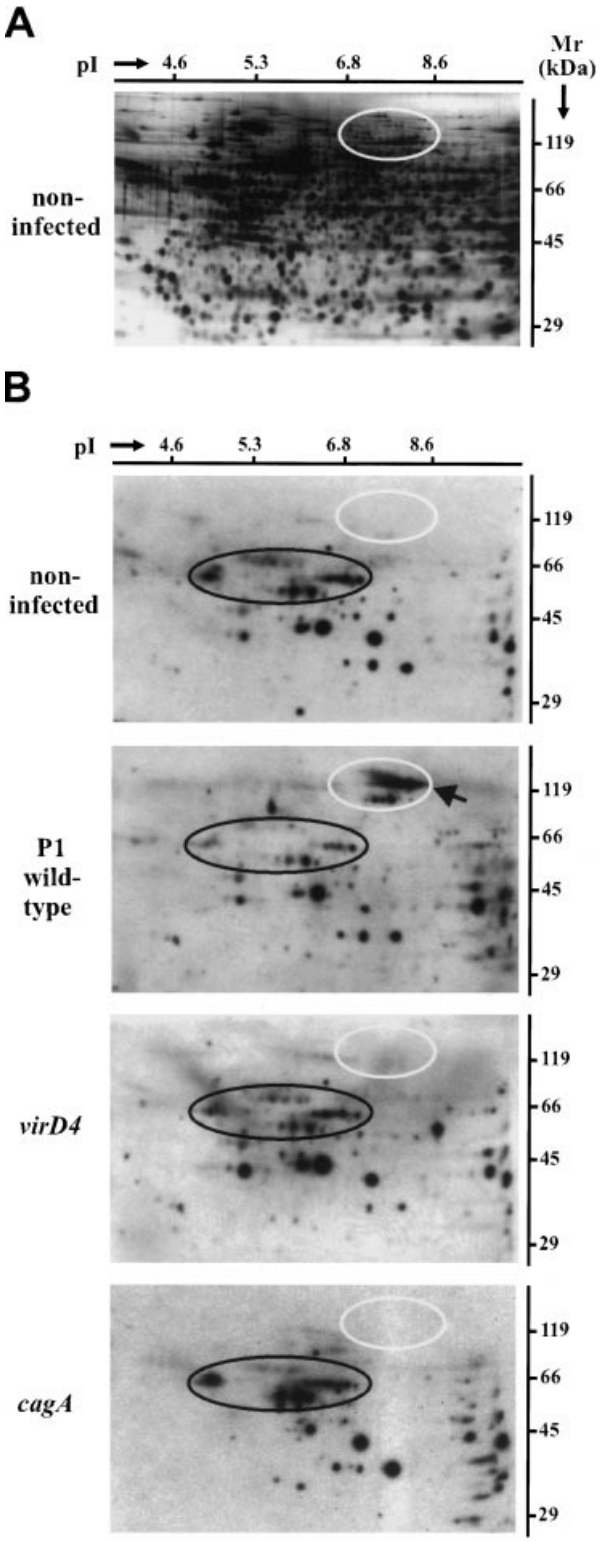

Fig. 4. Phosphorylation patterns of proteins isolated from *H. pylori*-infected AGS cells analysed by two-dimensional gel electrophoresis (2-DE). Proteins were prepared from non-infected or AGS cells infected with *H. pylori* P1 wild-type, *virD4 and cagA* mutants.
A. Silver-stained gel of proteins from non-infected AGS cells.
B. Immunoblot analysis with an antiphosphotyrosine antibody. Several proteins were found to be phosphorylated and dephosphorylated during infection with the *H. pylori* wild-type strain. The white circle indicates a set of two major 100 and 130 kDa phosphorylated protein species. The arrow indicates the position of the CagA protein, as later confirmed with MALDI-MS (see Fig. 5). The black circle surrounds a group of several dephosphorylated protein species after infection with wild-type *H. pylori*. *H. pylori* infection was for 2 h using a MOI of 50. Results are representative of three independent experiments.

infection (MOI) of 50 for a 2 h infection and did not change significantly at MOIs of 100 or 200. Molecular weight differences of phosphorylated 125–135 kDa proteins observed in infections with different *H. pylori* wild-type strains suggested that this protein could represent a phosphorylated bacterial protein rather than a host protein. The observation that the isogenic mutant of *cagA* abolished the presence of the respective 125 kDa protein and that CagA is not a proposed membrane component of the type IV secretion apparatus (Covacci *et al.*, 1999) led to the assumption that CagA is a bacterial protein translocated in the host cell. Reprobing of the blot with an anti-CagA antibody confirmed that one of the phosphorylated 125–135 kDa proteins represents the CagA protein (Fig. 2, bottom). CagA proteins are also present in attached *H. pylori* of strain J99 and *vir* gene mutants, but exclusively in the non-phosphorylated form (Fig. 2, compare top and bottom panels). As a control, *H. pylori* without cell contact does not show phosphorylated CagA, suggesting that host cell contact is a prerequisite for CagA phosphorylation (Fig. 2, top). These observations are consistent with the idea that CagA is phosphorylated only during infections of host cells with *H. pylori* strains encoding functional genes of the proposed type IV secretion machinery. However, we cannot rule out the existence of a polar effect in some of our *vir* mutants. The motility of some of the mutants was slightly reduced, but none of these mutations significantly affected the ability of the bacterium to adhere to host cells, or the production of the CagA protein (Fig. 2, bottom).

In a time course, phosphorylation of CagA appeared 15 min after attachment and was strongly established after 1 h accompanied by a strong dephosphorylation of the 75–80 kDa proteins (Fig. 3, top). Reprobing of this blot with an anti-CagA antibody showed gradually increasing amounts of the CagA protein present during

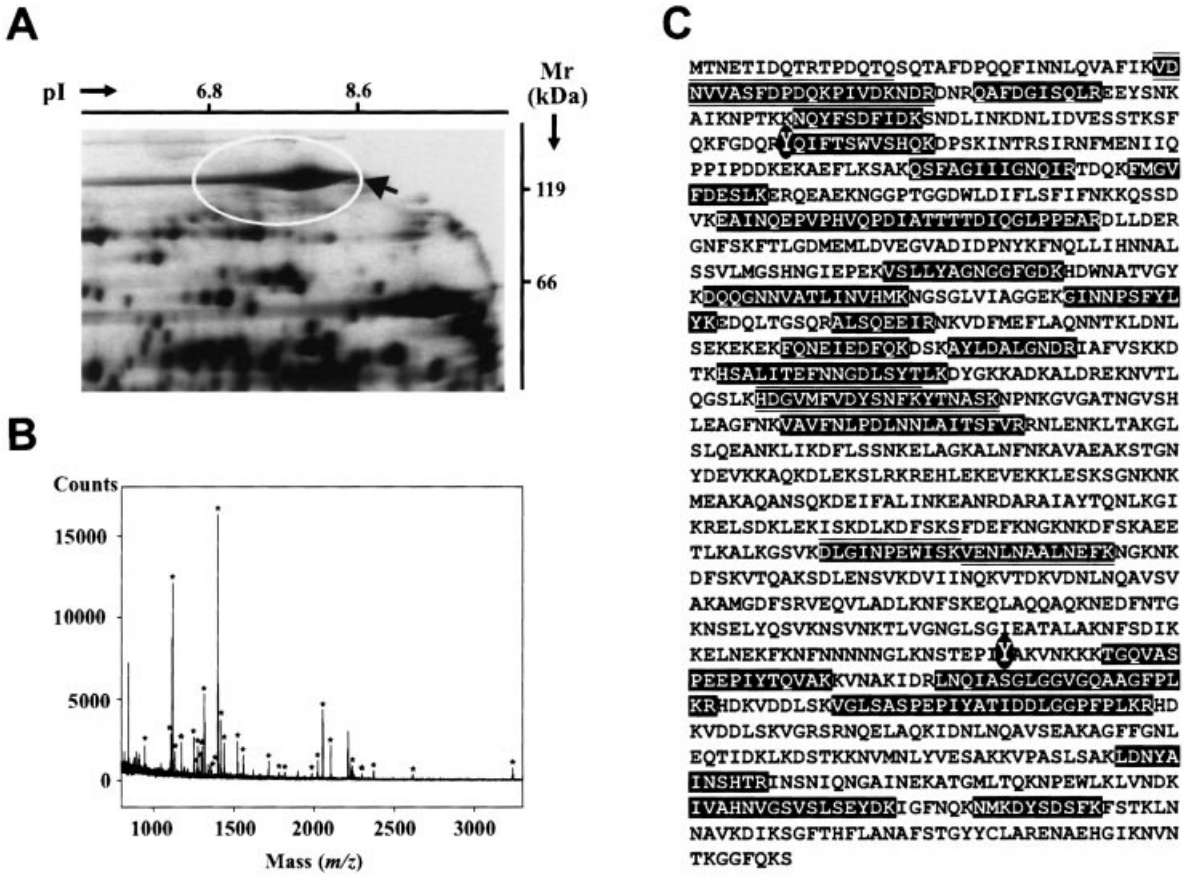

Fig. 5. Identification of CagA protein by two-dimensional gel electrophoresis (2-DE) and MALDI-MS.
A. Silver-stained gel of proteins from *H. pylori* strain P1. The arrow indicates the position of the CagA protein, as confirmed with MALDI-MS.
B. CagA MALDI-MS. Peaks marked with an asterisk were matched against the *H. pylori* database and correspond to CagA.
C. Amino acid sequence of the 132.4 kDa CagA protein from *H. pylori* strain 26695 (ORF 547; Tomb *et al.*, 1997; accession no. P55980). Peptide sequences identified by MALDI-MS after digestion with trypsin are highlighted in black. Overlapping peptide sequences are marked with bars. Two potential tyrosine phosphorylation sites at positions 116–123 and 892–900 have been determined with the Prosite database using the HUSAR program (http://www.genius.embnet.dkfz-heidelberg.de). Tyrosine residues in these motifs are shadowed with a black circle.

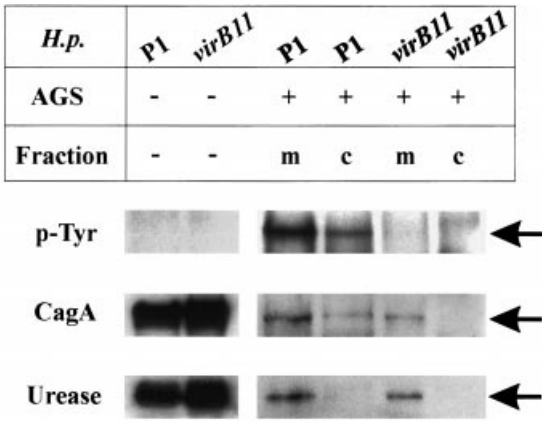

Fig. 6. Translocation of CagA into the host cell. Fractionation of membrane (m) and cytoplasmic (c) proteins after infection of AGS cells with *H. pylori* wild-type strain P1 and the isogenic *virB11* mutant was analysed by SDS–PAGE. Identification of the phosphorylated CagA protein with an antiphosphotyrosine antibody (top). Identification of CagA with an anti-CagA serum (middle). The blot shown in the middle was reprobed with an antibody against *H. pylori* urease (β-subunit) as a control (bottom). AGS cells were challenged with a MOI of 100 for 2 h. Total proteins isolated from *H. pylori* strain P1 and the *virB11* mutant served as a control. Representative results of three independent experiments are shown.

infection, which is probably the result of an increasing number of attached bacteria and translocated CagA during infection (Fig. 3, bottom). Incubation of AGS cells with the supernatant of the *H. pylori* culture did not induce changes in the tyrosine phosphorylation pattern of proteins, suggesting that CagA is not secreted into the medium.

Identification of CagA by two-dimensional gel electrophoresis and matrix-assisted laser desorption/ ionization mass spectrometry

To investigate protein tyrosine phosphorylation and dephosphorylation events of infected AGS cells in more detail and to confirm the identification of CagA by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) peptide mass fingerprinting, we compared two-dimensional gel electrophoresis (2-DE) phosphotyrosine patterns of non-infected and infected AGS cells after removal of the attached bacteria. Figure 4A shows a silver-stained gel of AGS cell proteins to which quantities of protein samples isolated from AGS cells infected with wild-type *H. pylori* strain P1 or mutants, such as *virD4* or *cagA*, have been standardized. Respective 2-DE blots probed with an anti-phosphotyrosine antibody are shown in Fig. 4B. These blots revealed several significant changes with respect to the phosphorylation status of proteins during host cell infection. In the complete scenario, we detected about six major differences. Most of them were composed of at least four completely or partially dephosphorylated proteins (black circle), and two major 100 and 130 kDa phosphorylated protein species appeared only after infection with wild-type *H. pylori* (white circle).

To identify possible *H. pylori* proteins on these patterns, we compared them with the silver-stained 2-DE protein expression patterns of *H. pylori* wild-type strains P1 (Fig. 5A), 26659, J99 and *cagA* mutant (data not shown). Overlays of these images revealed one major difference between several wild-type strains and the *cag* mutant: only the wild-type strains gave rise to a 130 kDa protein (compare Figs 4B and 5A, see arrow). We excised this protein from the Coomassie-stained gel and identified more than 30 peptides (marked with asterisks) by in-gel tryptic digestion followed by MALDI-MS (Fig. 5B). All peptides represented amino acid sequences of the *H. pylori* CagA protein, giving rise to a sequence coverage of 32%. The position of the peptides in the complete CagA protein sequence is shown in Fig. 5C. Two potential tyrosine phosphorylation sites in the CagA sequence are shadowed with a black circle. Our experiments finally demonstrate that one of the 125–135 kDa phosphorylated proteins is indeed CagA. The lack of signals on 2DE blots during infection with the *H. pylori vir* mutants, such as *virD4* (Fig. 4B, white circles), supports the hypothesis that translocation into the host cell is required for phosphorylation of the CagA protein.

Translocation of CagA into the host cell membrane and cytoplasm

To confirm our hypothesis and to identify the CagA protein within cells, AGS cells were infected with *H. pylori* wild-type strain P1 and its *virB11* mutant for 2 h followed by the separation of membrane as well as cytoplasmic fractions. The 130 kDa CagA protein was identified by immunoblot analysis with an anti-phosphotyrosine antibody and the anti-CagA antibody (Fig. 6, top). Phosphorylated CagA protein is present in the membrane as well as in the cytoplasm during infection with wild-type *H. pylori*. In contrast, the isogenic *virB11* mutant as an important key protein of the type IV secretion apparatus (Fig. 1) did not reveal phosphorylated CagA protein at all. This membrane fraction contained CagA as a result of bacteria attached to the host membrane, but in lower amounts. As a control, CagA protein prepared from *H. pylori* without host cell contact was not phosphorylated. To verify that the presence of CagA in the host is not a preparation artifact, the blot was reprobed with an antibody against *H. pylori* urease (Fig. 6, bottom). Urease is a protein that is secreted into the medium in high amounts, but is also present in the bacterial cytosol (data not shown). This protein has never been shown to be translocated into host

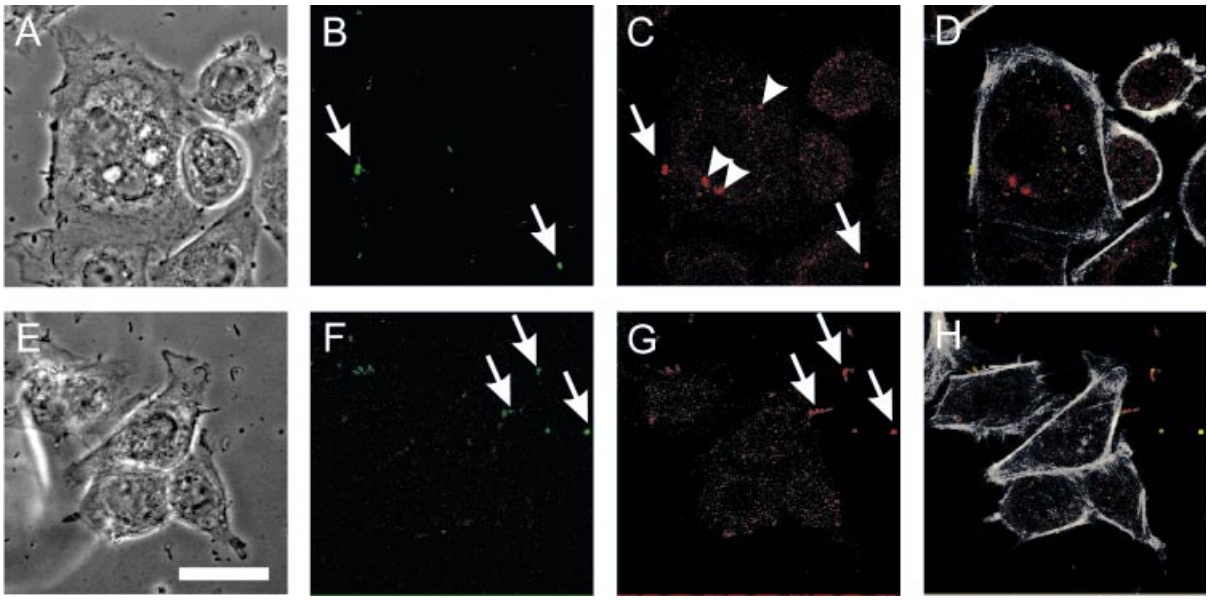

Fig. 7. Identification of CagA in the host membrane and cytoplasm by laser-scanning confocal immune fluorescence microscopy. *H. pylori* wild type (top, A–D) and *virB11* mutant (bottom, E–H) attached to AGS cells were used to localize CagA protein in the host cell.
A and E. Phase-contrast microscopy.
B and F. Fields stained for *H. pylori*.
C and G. Fields stained for CagA.
D and H. Merged image showing staining for *H. pylori* (green), CagA (red) and actin (white).
Arrows indicate co-localization of *H. pylori* and CagA (yellow) in the membrane. Arrowheads indicate CagA protein accumulations in the cytoplasm. For better visualization, grey values of confocal images were depicted as pseudocolours. Scale bar = 25 μm. AGS cells were challenged with a MOI of 100 for 2 h. Results are representative of three independent experiments.

cells. We observed cross-contamination with urease in the membrane because of attached bacteria but no cross-contamination in the host cytosol, suggesting that the presence of CagA protein in the host membrane and cytoplasmic fractions is caused by translocation of the protein from the bacterium into the host.

To visualize CagA protein directly in the host cell and to confirm its translocation from the bacterium into the host membrane and cytoplasm, confocal microscopy was applied. AGS cells were exposed to *H. pylori* wild-type strains and *virB4*, *virB7*, *virB10*, *virB11*, *virD4 and cagA* mutant strains. CagA from type I wild-type strains was detected inside or immediately beneath attached bacteria (Fig. 7B–D, arrows) as well as in the cytoplasm of host cells (Fig. 7C and D, arrowheads). Although intracellular *H. pylori* have been observed sporadically, signals of accumulated CagA in the host cytoplasm (as indicated by the arrowheads) did not show co-localization with bacteria. In contrast, CagA from strains mutated in *vir* genes, as important components of the type IV secretion apparatus, was exclusively located in bacterial cells, suggesting that translocation was blocked or greatly reduced (Fig. 7F and G). Non-infected AGS cells were devoid of the CagA signal, and type II strain 1061 lacking the complete *cag* PAI or *cagA* mutant showed no CagA signal, as expected (data not shown).

Discussion

The delivery of bacterial virulence factors into the host by a type IV secretion apparatus has been well described for the agrobacterial T-DNA and *B. pertussis* toxin export systems (for reviews, see Winans *et al*., 1996; Christie, 1997). By comparison, very little is known about the function of the newly discovered type IV secretion systems in *Legionella pneumophila* (Vogel and Isberg, 1999), *Brucella suis* (O'Callaghan et al., 1999), *Rickettsia prowazekii* (Andersson *et al*., 1998) or *Helicobacter pylori* (Covacci *et al*., 1999). The present data provide several lines of evidence for a function of the *H. pylori* type IV secretion machinery in actively translocating an important virulence determinant, CagA, into the host cell. Although no function for this immune-dominant antigen has been reported so far, we made the following observations that may deepen our understanding of the function of the *H. pylori* virulence system. (i) The CagA protein is tyrosine phosphorylated during infection with cultured gastric cells in a strain- and time-dependent manner. The CagA protein is not tyrosine phosphorylated in the *H. pylori* cells without host cell contact. Only several type I, but no type II, wild-type strains were able to induce CagA phosphorylation. (ii) CagA was not secreted into the medium, as supported by the observation that supernatants of *H.*

pylori type I strains did not induce changes in the phosphotyrosine pattern of infected AGS cells. (iii) We have shown the insertion of CagA into the membrane (at positions at which *H. pylori* is attached) and the cytosol of the host by two independent experimental approaches including fractionation studies and confocal microscopy. (iv) Injection and tyrosine phosphorylation of CagA in the host cell appears to be dependent on important functional core components (*vir* genes) and the *cagA* gene of the *H. pylori* type IV secretion apparatus encoded by the *cag* PAI (compare Fig. 1). (v) Translocation and phosphorylation of the CagA protein is accompanied by additional tyrosine phosphorylation and dephosphorylation events of several proteins in the host.

To facilitate studies of the *H. pylori*-induced signalling processes that underlie *cag*-dependent mechanisms, we have produced a set of *cag* mutants. The *cag* mutants were from strain P1, a type I strain that actually encodes all the putative homologues of the agrobacterial *virB4*, *virB7*, *virB9*, *virB10*, *virB11* and *virD4* genes reported so far (Covacci *et al*., 1999), and provokes a very strong host response. We have produced mutants from all these genes, except the *virB9* gene; although present in P1, we were not yet able to produce a functional mutant. However, all the open reading frames (ORFs) tested were needed for successful translocation and phosphorylation of the *H. pylori* CagA protein. Although we cannot completely rule out the occurrence of polar effects on downstream genes as a result of the integrated Cm^R cassette in some of these mutants, the protein expression patterns and the patterns of tyrosine-phosphorylated proteins of infected AGS cells in 2-DE were consistently observed with different MOIs similar to that produced by type II strains lacking the complete *cag* PAI. Moreover, our *vir* mutants were not significantly reduced in their ability to attach to the host cell when compared with its wild type, and also did not significantly affect the expression level of the CagA protein. However, our results, i.e. (i) the inability of the *H. pylori vir* gene mutant strains to translocate and phosphorylate the CagA protein, and (ii) the homology of these *vir* genes to that of *A. tumefaciens* or *B. pertussis*, are collectively consistent with a model in which different proteins encoded in the *cag* PAI form a contact-dependent and type IV-like secretion apparatus responsible for direct injection of CagA into the host cell (Fig. 1). Our results led us to suggest that intact *virB4*, *virB7*, *virB10*, *virB11*, *virD4*, *cagA* and possibly also *virB9* gene products are essential in the injection process of CagA.

Our data considerably extend those of Segal *et al*. (1999), who first presented evidence of CagA phosphorylation during the infection process. These authors showed that CagA is necessary to induce a growth factor-like phenotype (hummingbird) in host gastric cells and another CagA-independent cellular phenotype called SFA (stress fibre associated). We have also identified phosphorylated CagA protein by several approaches, including MALDI-MS. In addition, searches in the Prosite database revealed two potential tyrosine phosphorylation sites in the CagA sequence of strain 26695. In another strain, J99, CagA is also present during infection, but not in the phosphorylated form. Alignments of CagA protein sequences from strains J99 and 26695 revealed that CagA of J99 is mutated in respective tyrosine residues, which supports the view that both sites detected in strain 26695 could indeed have a function *in vivo* (unpublished data). This conclusion is supported by our observation that, only in infections with *H. pylori* type I strains showing phosphorylated CagA protein, were dephosphorylated 75–80 kDa proteins additionally observed in a time-dependent manner. The potential role of these tyrosine phosphorylation sites in the infection process and possibly in gastric disease has to be elucidated in future studies.

The potential function of the translocated CagA protein in the host is unknown. Database searches of CagA sequences did not reveal significant homologies to any known proteins. Recent mutational studies have implicated several genes in the *cag* PAI in the induction of interleukin (IL)-8 but some, most strikingly *cagA*, were not involved (Tummuru *et al*., 1995; Censini *et al*., 1996; Li *et al*., 1999). By analogy with the enteropathogenic *Escherichia coli* Tir protein, which is secreted in the host membrane by the bacterial type III secretion machinery and subsequently tyrosine phosphorylated, thereafter acting as a receptor for intimin binding on the bacterial surface (Kenny *et al*., 1997), it is tempting to speculate that CagA could act as a receptor for *H. pylori* in a similar manner to attract additional bacteria to adhere. Alternatively, other unknown function(s) should also be considered, as CagA has also been detected in the host cytoplasm. As outlined above, translocation and tyrosine phosphorylation of CagA are temporally correlated with dephosphorylation of 75–80 kDa proteins. The nature and function of these host proteins is unknown as yet. It will be very interesting in future to study the correlation between the two events, which may shed new light on *H. pylori*-induced signalling processes in gastric disease.

In conclusion, our study has revealed that CagA is the first *H. pylori* protein shown to be translocated into the host. The present *in vitro* studies emphasize the importance of several agrobacterial *vir* gene homologues in the translocation and tyrosine phosphorylation of the *H. pylori* CagA protein, possibly by an active process via a type IV secretion apparatus encoded in the *cag* PAI. This event is temporally embedded in a scenario of additional protein tyrosine phosphorylation and dephosphorylation events within the host cell, which may be crucial for *H. pylori* virulence. We feel that further molecular dissection

of the *cag* PAI, as well as CagA translocation and tyrosine phosphorylation-dependent signalling processes, will significantly contribute to an understanding of *H. pylori* pathogenesis mechanisms.

Experimental procedures

Bacterial strains and mutagenesis

Type I *H. pylori* strains P1, P12 and P92 (Sydney strain SS1) are clinical isolates that have been described previously (Schmitt and Haas, 1994; Corthesy-Theulaz *et al*., 1996; Lee *et al*., 1997). *H. pylori* strain P210 is a clinical strain isolated from a patient with gastric cancer. The type II *H. pylori* strain 1061 was kindly provided by J. Bijlsma and H. Kusters (Department of Medical Microbiology, Vrije Universiteit Amsterdam, The Netherlands). Strains 26695 and J99 are *H. pylori* isolates obtained from TIGR and AstraZeneca respectively (Tomb *et al*., 1997; Alm *et al*., 1999). Isogenic P1 knock-out mutants have been constructed by insertion of a chloramphenicol resistance gene cassette (Cm^R, 1 kb *Bam*HI/*Bgl*II fragment of plasmid pTnMax1) in cloned *virB4* (P205), *virB7* (P206), *virB10* (P207), *virB11* (P208), *virD4* (P209) and *cagA* (P211) genes according to a standard protocol (Haas *et al*., 1993). Briefly, the genes of interest were amplified by polymerase chain reaction (PCR) using primers based on the sequences of the genes from strains 26695 and J99 in the AstraZeneca *H. pylori* genome database (http://www.astra-boston.com/hpylori/). The PCR products were cloned in the pGEM-T vector (Promega). After mutagenesis by insertion of the Cm^R cassette in the *vir* genes, 3–5 μg of supercoiled plasmid DNA was added to 1×10^8 bacteria ml^{-1} brain–heart infusion (BHI) medium. After incubation for 6 h, bacteria were grown on agar plates containing 4–6 μg ml^{-1} chloramphenicol to select for chloramphenicol-resistant transformants obtained after 4–5 days according to a standard procedure (Haas *et al*., 1993). Correct integration of the Cm^R cassette into the *H. pylori* chromosome by double cross-over recombination was confirmed by PCR. All *H. pylori* strains were grown on horse serum agar plates supplemented with vancomycin (10 μg ml^{-1}), nystatin (1 μg ml^{-1}) and trimethoprim (5 μg ml^{-1}) and, if necessary, with chloramphenicol (4–6 μg ml^{-1}). Incubation was at 37°C for 2 days in an anaerobic jar containing a gas mix of 5% O_2, 10% CO_2 and 85% N_2 (Oxoid).

Infection assays and immunoblot analysis

AGS cells (ATCC CRL 1739; a human gastric adenocarcinoma epithelial cell line) were grown in 25 cm^2 flasks with RPMI/10% FCS for 1 or 2 days respectively. The cells were washed once with PBS, and 4 ml of fresh medium was added to each flask. *H. pylori* (1×10^8) were resuspended in 0.5 ml of PBS and added to 2×10^6 AGS cells at a MOI of 50. *H. pylori* culture supernatant was obtained after completely pelleting bacteria at 3000 *g* for 10 min. After incubation in a 5% CO_2/95% air incubator for 1–3 h, AGS cells were washed once with PBS (containing 1 mM sodium vanadate) to remove non-adherent bacteria. Whole-cell lysates with attached bacteria were made by pelleting the cells at 600 *g* and resuspending the pellet in an equal amount of 2 × SDS lysis buffer (250 mM Tris-HCl, pH 8.0, 4% SDS, 20% glycerol, 0.002% bromophenol blue, 6% 2-mercaptoethanol). SDS–PAGE and electroblotting to Immobilon-P membranes were performed as described by the manufacturers (Millipore, Bio-Rad). Antibody binding and detection were performed with the ECL system (Amersham). Mouse monoclonal antiphosphotyrosine antibody PY99 was purchased from Santa Cruz Biotechnology. Polyclonal antiurease antibodies have been described recently (Gomez-Duarte *et al*., 1998). Polyclonal anti-CagA antibodies were produced by immunization of mice with a fusion protein containing a CagA polypeptide that corresponds to residues 110–769 of the CagA sequence reported recently (Tummuru *et al*., 1993).

Cellular fractionation

For the preparation of membrane/nuclear and cytoplasmic fractions of infected host cells, we followed a special protocol to remove adherent and non-adherent bacteria. For this purpose, 2×10^6 infected cells were washed with PBS (containing 1 mM sodium vanadate) to remove non-adherent bacteria. Adherent bacteria were detached from AGS cells by incubation with 2 ml of trypsin for 5 min. The following steps were carried out at 4°C with precooled solutions. PBS/10% FCS (4 ml) was added to the cells, followed by centrifugation at 600 × *g* for 5 min. The majority of the remaining bacteria was removed by several washing steps in PBS. The pellets with purified AGS cells were washed again in 1 ml of buffer [10 mM Tris-HCl, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 10% glycerol, 10 mM K_2HPO_4, 1 mM sodium vanadate, 10 mM NaF, 0.5 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulphonyl fluoride (PMSF)]. The cells were centrifuged again at 600 × *g* for 5 min, and the pellet was resuspended in 200 μl of buffer. Nonidet-P40 (2 μl) was added to the cells, gently mixed and incubated for 1 min on ice. Membrane/nuclear fractions were collected by pelleting at 1000 *g* for 10 min. Cytoplasmic fractions were purified from the remaining cell debris by centrifugation at 12 000 *g* for 30 min. The fractions were resuspended in an equal amount of 2 × SDS lysis buffer.

Two-dimensional electrophoresis (2-DE)

For the resolution of proteins isolated from infected AGS cells by 2-DE, we followed the protocol described above and removed adherent and non-adherent bacteria. The purified AGS cells were washed again with PBS and sonicated in the presence of proteinase and phosphatase inhibitors (0.5 mM PMSF, 2 μg ml^{-1} aprotinin, 2 μg ml^{-1} leupeptin and 1 mM sodium vanadate). Proteins from *H. pylori* strains P1, J99 and 26695 were isolated from bacterial pellets obtained after centrifugation at 2000 *g*. All protein fractions were treated with 9 M urea, 70 mM DTT and 2% Triton X-100 to obtain completely denatured and reduced proteins. For the resolution of proteins, we applied a 2-DE gel system (Jungblut and Seifert, 1990) in a 7 cm × 8 cm version. Protein (50 μg) was loaded to the anodic side of the isoelectric focusing (IEF) gel. The proteins were detected by silver staining (Jungblut and

Seifert, 1990), by staining with Coomassie brilliant blue R250 and by immunoblot analysis as described above.

Peptide mass spectrometry

For the identification of CagA protein, 300 μg of total protein extract was applied to large-scale 2-DE gel (20 cm × 30 cm) with a resolution power of about 5000 protein species. Respective spots were excised from the gel and subjected to in-gel tryptic digestion using a peptide-collecting device (Otto *et al*., 1996). The peptide solution was mixed with an equal volume of a saturated α-cyano-4-hydroxy cinnamic acid solution in 50% acetonitrile, 0.3% TFA, and 2 μl was applied to the sample template of a MALDI-MS (Voyager Elite; Perseptive), as described recently (Jungblut *et al*., 1999). Peptide mass fingerprints were searched using the program MS-FIT (http://prospector.ucsf.edu/ucsfhtml/msfit.htm), reducing the proteins of the NCBI database to the *H. pylori* proteins and to a molecular mass range estimated from 2-DE ± 20%, allowing a mass accuracy of 0.1 Da for the peptide mass.

Laser scanning confocal immunofluorescence (IF) microscopy

To localize CagA proteins directly during infection, AGS cells were exposed to *H. pylori* wild types, *vir* and *cagA* mutant strains. Monolayers of 2×10^4 AGS cells were grown overnight in 16-well Lab-Tek chambers (Nunc) and infected with *H. pylori* cells of the indicated strains at an MOI of 50. Three hours after infection, the cells were washed with PBS to remove non-adherent bacteria and fixed with 4% formaldehyde in PBS. The cultures were permeabilized with 0.1% Triton X-100 in PBS for 30 min and stained with antibodies against *H. pylori* [rabbit serum 1:100 (Dako)/anti-rabbit IgG-Alexa 594 (Molecular Probes)], CagA (mouse serum 1:200, anti-mouse IgG-Cy5; Jackson), and filamentous actin was visualized using phalloidin-AlexaFluor 488 (Molecular Probes). Specimens were analysed with a confocal microscope (Leica TCS-SP).

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