The *Helicobacter pylori* CagF protein is a type IV secretion chaperone-like molecule that binds close to the C-terminal secretion signal of the CagA effector protein

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Type IV secretion systems are common bacterial macromolecule transporters that have been adapted to various functions, such as effector protein translocation to eukaryotic cells, nucleoprotein transfer to bacterial or eukaryotic cells, and DNA transport into and out of bacterial cells. *Helicobacter pylori*, the causative agent of bacterial gastritis, peptic ulcers, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma, uses the Cag type IV secretion system to inject the CagA protein into host cells, thereby altering gene expression profiles and the host cell cytoskeleton. The molecular mechanism of CagA recognition as a type IV substrate is only poorly understood, but seems to be more complex than that of other type IV secretion systems. Apart from 14 essential components of the secretion apparatus, CagA translocation specifically requires the presence of four additional Cag proteins. Here we show that the CagA-binding protein CagF is a secretion chaperone-like protein that interacts with a 100 aa region that is adjacent to the C-terminal secretion signal of CagA. The interaction between CagA and CagF takes place at the bacterial cytoplasmic membrane, and is independent of a functional type IV secretion apparatus and other *cag*-encoded factors. Our data indicate that CagF binding precedes recognition of the C-terminal CagA translocation signal, and that both steps are required to recruit CagA to the type IV translocation channel.

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INTRODUCTION

Helicobacter pylori, a Gram-negative bacterium that efficiently colonizes the human stomach, is one of the most common infectious agents and can be considered as a model for microbial persistence (Blaser & Atherton, 2004). Persistent colonization is the outcome of a complex interaction pattern with host tissues and the host immune system, comprising both bacterial and host factors. Two of the most important bacterial factors that are thought to contribute to the development of *H. pylori*-associated diseases are the vacuolating cytotoxin VacA and the *cag* pathogenicity island (PAI), which encodes a type IV secretion system (Blaser & Atherton, 2004; Rieder *et al.*, 2005a). Infection with type I *H. pylori* strains, which are defined by the presence of these virulence factors, is correlated with the development of more severe forms of disease, such as peptic ulcers, mucosa-associated lymphoid

tissue (MALT) lymphoma and adenocarcinoma (Suerbaum & Michetti, 2002; Peek & Blaser, 2002).

Type IV secretion systems are used by several Gram-negative bacterial pathogens to inject proteins directly into the cytoplasm of host cells, thereby to modulate important cellular functions (Fischer *et al.*, 2002; Cascales & Christie, 2003; Christie *et al.*, 2005). The Cag type IV secretion system has been shown to translocate the bacterial CagA protein into host cells (Odenbreit *et al.*, 2000), where it is tyrosine-phosphorylated and subsequently induces cytoskeletal rearrangements that either require, or are independent of, tyrosine phosphorylation (reviewed in Bourzac & Guillemin, 2005; Backert & Meyer, 2006). Moreover, CagA translocation results in signal transduction events that lead, for example, to proliferation defects in B lymphocytes or epithelial cells (Umehara *et al.*, 2003; Yokoyama *et al.*, 2005). Another cellular response caused by the Cag secretion system is the production and secretion of interleukin-8 (IL-8) by epithelial cells, which is independent of the CagA protein in some *H. pylori* strains but considerably enhanced by CagA in others (Fischer *et al.*, 2001b; Viala *et al.*, 2004; Brandt *et al.*, 2005). In the

Abbreviations: GFP, green fluorescent protein; GSK, glycogen synthase kinase; GST, glutathione S-transferase; IL-8, interleukin-8; PAI, pathogenicity island.

Mongolian gerbil model, the Cag system and the CagA protein have been implicated in the development of a corpus-predominant gastritis, which is considered to be a precancerous condition (Rieder *et al.*, 2005b). One process involved might be the CagA-dependent activation and nuclear accumulation of β -catenin (Franco *et al.*, 2005).

The molecular mechanisms of CagA translocation are only poorly understood. Since the Cag type IV secretion system is only distantly related to prototypical systems, such as the VirB/D4 system of *Agrobacterium tumefaciens*, considerable differences in the mechanistic details of translocation are likely to occur. Recent studies have shown that most type IV-secreted effector proteins have C-terminal secretion signals that are necessary and sufficient for translocation, although recognition of these signals may be modulated by the presence of other domains (Vergunst *et al.*, 2005; Schulein *et al.*, 2005; Nagai *et al.*, 2005). In contrast, the C-terminal CagA region is not sufficient to direct CagA translocation, although it has similar properties (Hohlfeld *et al.*, 2006). Recognition of effector proteins as type IV substrates and their recruitment to the secretion apparatus are thought to be achieved by the so-called coupling proteins (Gilmour *et al.*, 2003; Llosa *et al.*, 2003; Atmakuri *et al.*, 2003). Apart from the secretion apparatus and the coupling protein, additional factors are required for translocation of a subset of effector proteins. For example, the stability and secretion of the *A. tumefaciens* VirB/D4 effector protein VirE2 both depend on the secretion chaperone VirE1 (Sundberg *et al.*, 1996; Sundberg & Ream, 1999; Deng *et al.*, 1999; Zhou & Christie, 1999; Zhao *et al.*, 2001), although other VirB/D4 effector proteins do not seem to require secretion chaperones. Likewise, cytosolic complexes of the IcmW, IcmS and LvgA proteins, which are also considered to be chaperone-like molecules, play an important role in type IV translocation of a subset of Icm/Dot effector proteins in *Legionella pneumophila* (Ninio *et al.*, 2005; Bardill *et al.*, 2005; Vincent & Vogel, 2006). In type III secretion systems, which are also able to directly inject effector proteins into target cells, secretion chaperones function by maintaining effector protein stability, preventing premature interactions, or contributing to translocation channel targeting (Parsot *et al.*, 2003; Ghosh, 2004). It has been suggested that chaperone-dependent effector proteins are translocated with a higher priority in these systems.

The Cag type IV secretion system contains 18 gene products that are essential for CagA translocation. However, only 14 gene products are thought to encode components of the secretion apparatus, since besides their involvement in CagA translocation they are necessary for induction of IL-8 secretion from epithelial cells (Fischer *et al.*, 2001b). The four additional proteins that are specifically necessary for CagA translocation include the coupling protein homologue Cag β (HP524) and the CagF (HP543) protein, which has been shown to interact with CagA in the bacterial cell (Couturier *et al.*, 2006). Here we show that CagF functions as a secretion chaperone-like molecule that binds at the

bacterial cytoplasmic membrane to a region near the C terminus of the effector protein CagA and thus adjacent to its C-terminal translocation signal.

METHODS

Bacterial strains, cell lines and culture conditions. *H. pylori* strains were grown on GC agar plates (Remel) supplemented with vitamin mix (1%), horse serum (8%), vancomycin (10 mg l⁻¹), trimethoprim (5 mg l⁻¹) and nystatin (1 mg l⁻¹) (serum plates), and incubated for 16–40 h in a microaerobic atmosphere (85% N₂, 10% CO₂, 5% O₂) at 37 °C. *Escherichia coli* strains Top10 (Invitrogen) and DH5 α (BRL) were grown on Luria–Bertani (LB) agar plates or in LB liquid medium (Sambrook *et al.*, 1989) supplemented with ampicillin (100 mg l⁻¹), chloramphenicol (30 mg l⁻¹) or kanamycin (40 mg l⁻¹), as appropriate. AGS gastric carcinoma cells were cultivated under standard conditions, as described previously (Odenbreit *et al.*, 2000).

Plasmid constructs. Plasmids pJP89A, pSO174 and pJP55, which were used to generate isogenic *cagY* or *cagF* mutants, or to express *cagA* from a shuttle plasmid (Table 1), have been described previously (Odenbreit *et al.*, 2000; Fischer *et al.*, 2001b). For the construction of a *gsk*–*cagF* fusion, *cagF* without a start codon was amplified by PCR from chromosomal DNA of *H. pylori* strain 26695, using oligonucleotides WS215 and WS216 (Table 2), and cloned into the *Bgl*II and *Kpn*I sites of plasmid pWS231 (Hohlfeld *et al.*, 2006), resulting in plasmid pIP15. Plasmid pWS214 was constructed by cloning the same PCR fragment into plasmid pWS210 (Hohlfeld *et al.*, 2006) and subsequent subcloning of the *m45*–*cagF* gene fusion into the *Cl*aI and *Sac*I sites of the shuttle vector pHel3. Plasmid pIP31 was constructed by cloning a DNA fragment obtained by PCR with primers WS286 and WS287 into the *Spe*I and *Bgl*II sites of plasmid pWS122, and subsequent subcloning of the *cagF*–*gfp* fusion into the *Sal*I and *Kpn*I sites of the chromosomal integration vector pJP99. The construction of plasmids pIP8, pIP9, pIP9-2, pIP22, pWS231 and pWS252, encoding glycogen synthase kinase (GSK)-tagged CagA variants, and of plasmid pWS159-21 encoding a C-terminal CagA truncation variant, has been described previously (Hohlfeld *et al.*, 2006). Plasmid pWS274 was constructed by PCR amplification of a *gsk*–*cagA* fragment from plasmid pIP9 using primers WS158 and WS318 and cloning into the *Cl*aI and *Bam*HI sites of pWS231. For the production of a glutathione S-transferase (GST)–CagF fusion protein, the *cagF* gene was amplified by PCR from strain 26695 using primers WS221 and WS222, and cloned into the *Bam*HI and *Xho*I sites of pGEX4T-3 (Amersham Pharmacia Biosciences), resulting in plasmid pWS226. Similarly, the *cagA* gene was amplified from strain P12 using primers WS315 and HK3, and cloned into the *Bam*HI and *Xho*I sites of pGEX4T-3, resulting in plasmid pRL1. For the construction of plasmid pIP32, the *gsk*–*cagF* fusion was subcloned from plasmid pIP15 into the *Eco*RI and *Kpn*I sites of pCDFDuet-1 (Novagen). Plasmids pRL2 and pRL4, encoding fusions of green fluorescent protein (GFP) to the C-terminal 50 and 100 amino acids of CagA, respectively, were constructed by PCR amplification of 3' *cagA* regions using primer pairs WS316/JP67 or WS321/JP67, and cloning into the *Bgl*II and *Kpn*I sites of plasmid pWS130. Plasmid pWS273, encoding GFP–CagA-195C, was obtained by cloning a 3' *Bgl*II/*Kpn*I *cagA* fragment into the same sites of pWS130, and plasmid pRL3 is a *Bgl*II/*Bam*HI deletion derivative of plasmid pWS130.

Bacterial transformation and conjugation. Shuttle plasmids and suicide plasmids were introduced into *H. pylori* strains by conjugation or transformation, as described previously (Haas *et al.*, 1993; Fischer & Haas, 2004). *H. pylori* transformants were selected on serum agar plates containing 6 mg chloramphenicol l⁻¹ or 8 mg kanamycin l⁻¹.

Table 1. Plasmids used in this study

Plasmid	Relevant characteristics	Source or reference
pGEX4T-3	<i>gst</i> fusion expression vector	Amersham Pharmacia
pHel2	Shuttle vector	Heuermann & Haas (1998)
pHel3	Shuttle vector	Heuermann & Haas (1998)
pIP8	pHel2, <i>gsk-cagA</i> (codons 1020–1214)	Hohlfeld <i>et al.</i> (2006)
pIP9	pHel2, <i>gsk-cagA</i>	Hohlfeld <i>et al.</i> (2006)
pIP9-2	pHel2, <i>gsk-cagA</i> (codons 1–702)	Hohlfeld <i>et al.</i> (2006)
pIP15	pHel2, <i>gsk-cagF</i>	This study
pIP22	pHel2, <i>cagA</i> (codons 1–613)– <i>gsk</i>	Hohlfeld <i>et al.</i> (2006)
pIP31	pJP99, <i>cagF-gfp</i>	This study
pIP32	pCDFDuet-1, <i>gsk-cagF</i>	This study
pJP55	pHel2, <i>cagA</i> (<i>H. pylori</i> 26695)	Odenbreit <i>et al.</i> (2000)
pJP89A	pBA, Δ <i>hp527::cat</i>	Fischer <i>et al.</i> (2001b)
pJP99	Chromosomal integration plasmid	Püls <i>et al.</i> (2002)
pRL1	pGEX4T-3, <i>cagA</i>	This study
pRL2	pHel2, <i>gfp-cagA</i> (codons 1164–1214)	This study
pRL3	pHel2, <i>gfp</i>	This study
pRL4	pHel2, <i>gfp-cagA</i> (codons 1114–1214)	This study
pSO174	pBA, Δ <i>hp543::cat</i>	Fischer <i>et al.</i> (2001b)
pWS122	pHel2, <i>cagA'-gfp</i>	Hohlfeld <i>et al.</i> (2006)
pWS130	pHel2, <i>gfp-cagA</i> (codons 614–1214)	Hohlfeld <i>et al.</i> (2006)
pWS159-21	pJP99, <i>cagA</i> (codons 1–1122)	Hohlfeld <i>et al.</i> (2006)
pWS210	pHel2, <i>m45-cagA</i> (codons 614–1214)	Hohlfeld <i>et al.</i> (2006)
pWS214	pHel3, <i>m45-cagF</i>	This study
pWS226	pGEX4T-3, <i>cagF</i> (<i>H. pylori</i> 26695)	This study
pWS231	pHel2, <i>gsk-cagA</i> (codons 614–1214)	Hohlfeld <i>et al.</i> (2006)
pWS252	pHel2, <i>gsk-cagA</i> (codons 1–1194)	Hohlfeld <i>et al.</i> (2006)
pWS273	pHel2, <i>gfp-cagA</i> (codons 1019–1214)	This study
pWS274	pHel2, <i>gsk-cagA</i> (codons 1–868)	This study

Production of GST fusion protein and GST pulldown assays.

For the production and purification of GST–CagF fusion protein, overnight cultures of *E. coli* strain BL21 (DE3) containing plasmid pWS226 or pGEX4T-3 as a control were diluted in fresh LB medium and grown for 4 h at 37 °C in a shaking incubator. Expression was induced by addition of IPTG to a final concentration of 0.2 mM, and

cells were grown for an additional 2 h. Bacterial cells were harvested by centrifugation, resuspended in 50 mM Tris/HCl, pH 7.4, containing protease inhibitors (1 mM PMSF, 10 µg leupeptin ml⁻¹, 10 µg pepstatin ml⁻¹), and lysed by two passages through a French pressure cell. The lysate was centrifuged for 15 min at 15 000 g to remove insoluble material, and the supernatant was subjected to affinity chromatography on glutathione Sepharose 4B (GE Amersham Pharmacia) according to the manufacturers' instructions. The GST fusion protein was allowed to bind to the affinity matrix for 30 min at room temperature, and after three washes of the matrix with 10 bed volumes each of PBS, it was eluted in three fractions with 50 mM Tris/HCl, pH 8.2, 30 mM reduced glutathione for 15 min each at room temperature.

Table 2. Oligonucleotide primers used in this study

Primer	Sequence (5'–3')*
HK3	CGGGATCCGTCGACTTAAGATTTTGGAAACCAC
JP67	GGCCGAGCTCGGTACCTTAAGATTTTGGAAACC-AC
WS158	CCATCGATGGTAAAAATGTGAATCGT
WS215	GAAGATCTTTTGC GCGAACA AAAAATTA
WS216	GGGGTACCTCAATCGTTACTTTTGTTT
WS221	CGGGATCCAAACAAAGTTTGC GCGAAC
WS222	ACCGCTCGAGTCAATCGTTACTTTTGTTT
WS286	GGACTAGTTAGTAAGGAGTATTAAAAAGAAAC
WS287	GAAGATCTCCATCGTTACTTTTGTTTGATT
WS315	CAGGATCCACTAACGAAACCATTAAACC
WS316	GCAGATCTCACCAACTTGAACAACGCTG
WS318	CGGGATCCGAAAAGTTTTAGAAAAG
WS321	GAAGATCTCATGCTAGCGCAAAAAACCC

*Restriction sites used for cloning are underlined.

For GST pulldown assays, glutathione Sepharose 4B was washed with PBS, then 200 µg purified GST or GST–CagF was added to a 100 µl bed volume of glutathione Sepharose and incubated at room temperature for 1 h. Excess protein was removed by three washes with PBS containing protease inhibitors. Cleared bacterial lysates obtained by sonication and subsequent centrifugation were added and the mixture was incubated overnight at 4 °C. After three washes with PBS, GST fusions and bound proteins were eluted three times with 50 mM Tris/HCl, pH 8.0, containing 10 mM reduced glutathione, and analysed by immunoblotting.

Antisera, SDS-PAGE and immunoblotting. For the generation of the rabbit polyclonal CagF antiserum AK284, the purified GST–CagF fusion protein was proteolytically processed for 12 h at 4 °C with thrombin (1000 U; GE Amersham Pharmacia). CagF was subsequently separated from GST and thrombin by chromatography on

benzamidine Sepharose (GE Amersham Pharmacia) and glutathione Sepharose 4B according to the manufacturer's instructions, and recovered in 50 mM Tris/HCl, pH 7.4, containing 150 mM NaCl. The pooled fractions containing purified CagF were used to raise the rabbit antiserum AK284. Antisera AK257, directed against a C-terminal CagA domain, AK263, directed against *H. pylori* RecA, and anti-CagX (JHP477) have been described previously (Odenbreit *et al.*, 2000; Fischer & Haas, 2004). The GST antiserum was obtained from Sigma (monoclonal anti-GST, clone GST-2). For immunoprecipitation, antisera were purified on protein G-Sepharose, as described previously (Rohde *et al.*, 2003).

SDS-PAGE and Western blotting were performed as described previously (Fischer *et al.*, 2001b). For visualization of proteins after SDS-PAGE, gels were stained with Coomassie brilliant blue R250 or with silver stain (Blum *et al.*, 1987). For the development of immunoblots, PVDF filters were blocked with 3% BSA in TBS (50 mM Tris/HCl, pH 7.5, 150 mM NaCl) and incubated with the respective antisera at a dilution of 1:1000–1:5000. Alkaline phosphatase-conjugated protein A or horseradish peroxidase-conjugated anti-rabbit IgG was used to visualize bound antibody.

Immunoprecipitation. Bacteria grown on agar plates were suspended in PBS and washed twice. Bacteria (5×10^{10}) were resuspended in radioimmunoprecipitation (RIPA) buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM PMSF, 10 μ g leupeptin ml^{-1} , 10 μ g pepstatin ml^{-1}) and the cells were lysed by sonication. Unbroken cells were removed by centrifugation for 10 min at 10 000 g. A 5 μ l volume of the appropriate antiserum was added to the supernatant, and samples were incubated for 3 h at 4 °C. Then 50 μ l of prewashed protein G-agarose (Roche Diagnostics) was added and samples were incubated at 4 °C for an additional 2 h. After three washing steps with RIPA buffer, proteins were eluted with 100 mM glycine, pH 2.7, or by boiling in SDS-PAGE sample solution.

Determination of IL-8 secretion. The production of IL-8 by AGS cells after infection with *H. pylori* strains for 4 h was determined from cell supernatants by sandwich ELISA, as described previously (Fischer *et al.*, 2001b).

Tyrosine phosphorylation and GSK phosphorylation assays. Standard infections of AGS cells with *H. pylori* strains and subsequent preparation for phosphotyrosine immunoblotting were performed as described previously (Odenbreit *et al.*, 2000). Briefly, cells were infected with bacteria at an m.o.i. of 100 for 4 h at 37 °C. After examination of cell morphology by light microscopy, cells were washed three times and suspended in PBS containing 1 mM EDTA, 1 mM Na_3VO_4 , 1 mM PMSF, 10 μ g leupeptin ml^{-1} and 10 μ g pepstatin ml^{-1} . Cells with adherent bacteria were collected by centrifugation and resuspended in sample solution. For the determination of serine phosphorylation of the GSK tag, cells were infected and harvested as above, except that phosphatase inhibitor cocktail 1 (Sigma) was added to the suspension buffer. Tyrosine-phosphorylated proteins were analysed by immunoblotting with the phosphotyrosine antiserum PY99 (Santa Cruz Biotechnologies), and GSK phosphorylation was assayed by immunoblotting with phospho-GSK-3 β -specific antiserum 9336 (Cell Signaling Technology). As controls, the lysates were assayed for CagA production using antiserum AK257 (Odenbreit *et al.*, 2000), or for GSK tag production using the GSK-3 β -specific antiserum 9332 (Cell Signaling Technology). An *in vitro* GSK phosphorylation assay was performed as described previously (Hohlfeld *et al.*, 2006).

Analytical gel filtration chromatography. To determine the native molecular masses of CagF and the CagA–CagF complex, *E. coli* BL21 (DE3) containing either plasmid pWS226 or plasmids pRL1 and pIP32 was cultured and expression was induced as described above.

The GST–CagF fusion protein, or the complex of GST–CagA and GSK–CagF, was purified by affinity chromatography on glutathione Sepharose 4B, as described above, and CagF, or the complex of CagA and GSK–CagF, respectively, was released by thrombin cleavage and recovered in 50 mM Tris/HCl, pH 7.5, 150 mM NaCl. After high-speed centrifugation (45 000 g, 45 min), purified CagF was applied to a Superdex 75 column with a bed volume of 1.2 ml operated by a SMART chromatography system (Amersham Biosciences) and equilibrated with the same buffer. Beginning with the exclusion volume, 25 μ l fractions were collected and analysed by CagF immunoblotting. Molecular masses were estimated using a Gel Filtration LMW Calibration kit (Amersham Biosciences). Similarly, purified CagA/GSK–CagF was applied to a Superdex 200 column operated by a SMART system, and fractions were analysed by CagA and CagF immunoblotting. Molecular masses were estimated using a Gel Filtration HMW Calibration kit (Amersham Biosciences).

RESULTS

CagF is a translocation factor for CagA, but is not translocated by the type IV apparatus

We have previously shown that a *cagF* (*hp543*) mutant of *H. pylori* strain 26695 has a defect in CagA translocation but is still competent for induction of IL-8 from epithelial cells. Since the IL-8 phenotype is thought to depend on a functional type IV secretion apparatus, we concluded that CagF, like other *cag*-encoded proteins (CagI/HP540, CagZ/HP526 and Cag β /HP524), is not a component of the type IV secretion apparatus but is specifically required as a CagA translocation factor (Fischer *et al.*, 2001b). To confirm this result in a different strain background, we generated isogenic *cagF* and *cagY* mutants in *H. pylori* strain P12, a strain in which IL-8 induction is not completely independent of CagA translocation (Brandt *et al.*, 2005), and tested these mutants for their ability to induce IL-8 secretion and translocate CagA. As expected, the *cagY* mutant of strain P12, lacking an essential component of the Cag secretion apparatus, was defective in IL-8 induction from AGS cells (Fig. 1a). In contrast to the *cagF* mutant in strain 26695, the P12 *cagF* mutant showed a decreased ability to induce IL-8 secretion, reaching values of only about 65% of those of the wild-type strain. Consistently, IL-8 induction of an isogenic *cagA* mutant of strain P12 also reached levels of only about 62% of that of the wild-type (Fig. 1a). To investigate this phenotype further, we complemented the P12 Δ *cagF* mutant with plasmid pIP31, expressing a fusion of *cagF* and *gfp*. The complemented strain showed restored IL-8 induction levels (Fig. 1a). Furthermore, the P12 Δ *cagF* mutant was defective in CagA translocation to epithelial cells, as shown by CagA tyrosine phosphorylation, and this phenotype could also be restored by complementation with *cagF*–*gfp* (Fig. 1b). To confirm this finding, cells infected with the different strains were examined for induction of the hummingbird phenotype, a characteristic cell elongation which depends on the presence of phosphorylated CagA inside the epithelial cell (Segal *et al.*, 1999). The P12 Δ *cagF* mutant was unable to induce a hummingbird phenotype, whereas P12 Δ *cagF* complemented

Fig. 1. CagF functions as a CagA translocation factor but is not a type IV effector protein. (a) AGS cells were infected with *H. pylori* wild-type (wt) strain P12 and isogenic mutants lacking the *cagY*, *cagF* or *cagA* genes, and culture supernatants were assayed for IL-8 secretion using sandwich ELISA. Furthermore, the *cagF* mutant was complemented with plasmid pIP31, expressing a *cagF-gfp* fusion. All data are shown as mean and SD of at least three independent experiments. (b) Tyrosine phosphorylation assay of AGS cells infected for 4 h with P12, its isogenic *cagF* mutant, or the *cagF* mutant complemented with plasmid pIP31. CagA tyrosine phosphorylation was determined by immunoblotting [Western blotting (WB)] using the phosphotyrosine antiserum PY99. Induction of the hummingbird phenotype was assayed microscopically and is indicated as strong (++) or absent (-). (c) AGS cells infected with *H. pylori* strains producing GSK-tagged CagA or CagF proteins were lysed and assayed for the presence of translocated proteins by immunoblotting against the GSK tag and phosphorylated GSK. As a control to show the accessibility of the GSK tag in the GSK-CagF fusion, *H. pylori* and AGS cell lysates were mixed and incubated in an *in vitro* phosphorylation assay (ivP).

with pIP31 showed the same amount of elongated cells as the wild-type (Fig. 1b). These data clearly demonstrate that CagF is required for CagA translocation, but not for CagA-independent type IV-associated functions.

To exclude the possibility that CagF is co-translocated with CagA into epithelial cells and is required there for CagA tyrosine phosphorylation, we constructed a fusion of the phosphorylatable GSK-3 β reporter tag (Hohlfeld *et al.*, 2006; Torruellas-Garcia *et al.*, 2006) to the N terminus of CagF. Production of GSK-tagged proteins in *H. pylori* is assessed by immunoblotting using an anti-GSK-3 β antiserum, and translocation of these proteins into AGS cells by measuring serine phosphorylation of the GSK tag (Fig. 1c). While the GSK tag was phosphorylated in the GSK-CagA fusion protein after infection, no GSK phosphorylation could be detected in GSK-CagF. Since the GSK reporter tag is phosphorylated both at the cytoplasmic membrane and in the cytoplasm (Torruellas-Garcia *et al.*, 2006), and thus detects translocated proteins in both locations, this strongly suggests that CagF is not translocated to AGS cells.

CagF interacts with CagA independently of other translocation factors or the secretion apparatus

Translocation factors would be expected to interact with their cognate substrates, and possibly also with specific

secretion apparatus components. To identify proteins encoded on the *cag* PAI that interact with CagA, we immunoprecipitated CagA from total cell lysates of the wild-type *H. pylori* strain P12, its isogenic *cagA* deletion mutant and an isogenic strain lacking the complete *cag* PAI, but expressing *cagA* from a shuttle plasmid (pJP55). The only prominent protein that coprecipitated with CagA in the wild-type strain, but not in the *cagA* mutant or in the *cagA*-expressing Δ PAI mutant, had an apparent molecular mass of ~35 kDa (Fig. 2a). Immunoblotting identified this protein as CagF, which has been shown elsewhere to interact with CagA (Couturier *et al.*, 2006) (Fig. 2b). Since this interaction has also been found in a yeast two-hybrid assay (S. Kutter and W. Fischer, unpublished data), and can be demonstrated in *E. coli* by immunoprecipitation (Couturier *et al.*, 2006), it is a very strong and direct interaction. To test whether the interaction in *H. pylori* depends on the presence of other *cag*-encoded factors, notably the coupling protein homologue Cag β , we performed immunoprecipitation experiments on the *cagV* (*hp530*) mutant of strain 26695, with CagV representing an essential secretion apparatus component, and on the *cagI* (*hp540*), *cagZ* (*hp526*) and *cag β* (*hp524*) mutants, which lack genes encoding further CagA translocation factors (Fischer *et al.*, 2001b; Buhrdorf *et al.*, 2003). However, the amount of CagF coprecipitating with CagA was unchanged in these mutants (Fig. 2c, d and data not shown),

Fig. 2. CagF is a prominent CagA interaction partner in *H. pylori*. (a) In order to identify CagA-interacting proteins encoded on the *cag* PAI, CagA immunoprecipitation (IP) was performed from the wild-type strain P12, its isogenic *cagA* mutant and a P12 mutant expressing *cagA* from a shuttle plasmid, but lacking the complete PAI (P12ΔPAI[pJP55]). Silver staining shows the precipitation of CagA (arrowheads) and a coprecipitating PAI-encoded protein of about 35 kDa (arrow). (b) Western blotting (WB) with the polyclonal CagF antiserum AK284 identifies this protein as CagF. (c) Immunoprecipitation experiments in *H. pylori* strain 26695 and isogenic mutants. CagA was immunoprecipitated from the wild-type strain 26695 and isogenic mutants in the *cagA*, *cagV* and *cagF* genes. Starting extracts used for immunoprecipitation and immunoprecipitates were examined by Western blotting using CagA and CagF antisera. (d) Immunoprecipitation of CagA from strain 26695 and its isogenic *cagβ* mutant. Precipitates were examined by Western blotting using CagA and CagF antisera.

suggesting that the interaction is independent of a functional secretion apparatus and of the presence of translocation factors. Thus, CagF binding probably precedes recruitment of CagA to the secretion apparatus, which is consistent with a role for CagF as a chaperone-like molecule.

CagF is localized at the bacterial cytoplasmic membrane

In order to fulfil a secretion chaperone-like role, CagF would be expected to be localized in the bacterial cytoplasm. However, conflicting results have been obtained recently with respect to CagF localization in the cytoplasmic membrane or at the bacterial surface (Couturier *et al.*, 2006; Seydel *et al.*, 2002). Since we were unable to detect CagF at the bacterial surface by immunofluorescence using the CagF antiserum AK284 (data not shown), we analysed CagF localization in bacterial cell fractions. After sub-fractionation of *H. pylori* cells into membrane-bound and soluble proteins, we found CagF to be partly soluble and partly membrane-associated (Fig. 3a), as also shown elsewhere (Couturier *et al.*, 2006). The total membrane fraction was subsequently extracted with 1 % (w/v) Triton X-100, which is often used to solubilize cytoplasmic membrane proteins (Schnaitman, 1971; Nikaido, 1994). This procedure resulted in a partial release of CagF from the membrane fraction. An extraction behaviour similar to that of CagF was found for the cytoplasmic-membrane-associated protein CagX and for the integral cytoplasmic

membrane protein ComB8 (Hofreuter *et al.*, 2003), whereas the putative outer-membrane proteins CagX and AlpB could not be extracted with Triton X-100 (Fig. 3a). Comparable results were also obtained by membrane extraction with *N*-lauroyl sarcosine (data not shown), confirming the findings of Couturier *et al.* (2006). To examine whether CagF is an integral or a peripheral membrane protein, we extracted a total membrane fraction with 1 M NaCl. CagF and CagX were extracted by this procedure (Fig. 3b), whereas the integral cytoplasmic membrane protein ComB8 was not extracted under the same conditions. Taken together, the most likely localization of CagF is both in the cytoplasm and peripherally associated with the cytoplasmic membrane.

Since a *cagF*-*gfp* fusion was able to functionally complement the *cagF* mutation, as determined by the ability of the complemented strains to translocate CagA (Fig. 1b), it is likely that the localization of CagF-GFP reflects the actual localization of CagF in the bacterial cell. In order to determine this localization, we examined an *H. pylori* strain producing CagF-GFP by confocal microscopy. Apart from an even distribution in the bacterial cytoplasm, probably reflecting the cytoplasmic pool of CagF, GFP fluorescence was mostly found in several distinct patches close to the bacterial membrane (Fig. 3c, left panel). The same staining pattern was observed when *cagF*-*gfp* was expressed in a *cagA* mutant (data not shown), whereas GFP alone showed a uniform fluorescence (Fig. 3c, right panel). Furthermore, the strong GFP fluorescence in strain P12Δ*cagF* [pIP31] indicates that the CagF C terminus

Fig. 3. Localization of CagF in the bacterial cell. (a) Cells of *H. pylori* strain P12 were lysed and separated by ultracentrifugation into a soluble fraction containing cytoplasmic and periplasmic proteins (C/P) and a total membrane fraction which was subsequently extracted with 1% Triton X-100. Triton X-100-soluble proteins (Extr TX100) correspond to cytoplasmic membrane proteins, and Triton X-100-insoluble proteins (Pellet TX100) correspond to outer-membrane proteins. Immunoblots with the indicated antibodies (α -) were used to determine protein concentrations in the respective fractions. (b) To determine whether CagF is an integral or a peripheral membrane protein, the total membrane fraction was extracted with 1 M NaCl. The soluble fraction (C/P), the NaCl-extracted fraction (Extr NaCl) and NaCl-insoluble material (Pellet NaCl) were assayed by immunoblotting against the indicated proteins. (c) Confocal fluorescence microscopy of *H. pylori* cells producing a functional CagF-GFP fusion protein from plasmid pIP31 (left panel) or GFP alone from plasmid pRL3 (right panel).

(and therefore the complete protein) is localized at the cytoplasmic, and not the periplasmic, face of the inner membrane (Drew *et al.*, 2002).

CagF interacts with CagA at the cytoplasmic membrane, but is not responsible for CagA membrane targeting

Since both CagF and CagA are found in membrane-associated and cytoplasmic pools, we were interested in where their interaction takes place. To investigate this, we fractionated an *H. pylori* cell lysate by ultracentrifugation into a membrane fraction and a soluble fraction and performed immunoprecipitations from both fractions independently. Although CagA and CagF were found in comparable amounts in both fractions, CagF mainly co-precipitated with CagA from the membrane fraction, and only in minor amounts from the soluble fraction (Fig. 4a). This suggests that CagA and CagF form a stable complex associated with the bacterial cytoplasmic membrane. We speculated that CagF might bind to CagA in the cytoplasm and then recruit it to the membrane. To test this, we prepared membrane and soluble fractions of the isogenic *cagA* and *cagF* mutants in strain 26695 and compared them with the wild-type strain. As shown in Fig. 4(b), deletion of *cagF* had no influence on CagA membrane association, and deletion of *cagA* had no influence on CagF membrane association either, indicating that both proteins target the membrane independently and that CagF thus does not have a membrane recruitment function for CagA.

CagF forms homodimers and interacts with CagA as a complex of more than 200 kDa

Since secretion chaperones in type III secretion systems often form dimers, we employed gel filtration chromatography to determine the size of purified CagF. For that purpose, we overexpressed a *gst-cagF* fusion from plasmid pWS226 in *E. coli*, and purified GST-CagF by affinity chromatography on glutathione Sepharose 4B. CagF was eluted after cleavage of the GST moiety with thrombin, and applied to a Superdex 75 gel filtration column. From the gel filtration column, CagF eluted as two peaks centred at about 70 and 35 kDa, respectively, indicating that purified recombinant CagF exists in both a monomeric and a dimeric form (Fig. 5a). To estimate the size of the CagA-CagF complex, we coexpressed *gst-cagA* and *gst-cagF* in *E. coli* from plasmids pRL1 and pIP32, purified the complex formed by both proteins on glutathione Sepharose 4B and eluted the complex after thrombin cleavage. Size-exclusion chromatography was performed on a Superdex 200 column, and eluted fractions were tested for CagA and CagF content by immunoblotting. CagA eluted as a broad peak with the maximal concentration corresponding to an apparent molecular mass of 180–250 kDa (fractions 17–21; Fig. 5b). Minor amounts of CagA were also found in the range between 120 and 180 kDa and at higher molecular masses, suggesting that several different oligomeric complexes containing CagA were present. In many fractions, CagA was partially processed to a major breakdown product of about 100 kDa, which is frequently encountered

Fig. 4. Interaction of CagA and CagF takes place at the bacterial membrane, but CagF is not necessary for CagA membrane recruitment. (a) *H. pylori* cells were separated by ultracentrifugation into a total membrane fraction (TM) and a soluble fraction containing cytoplasmic and periplasmic proteins (C/P). CagA was immunoprecipitated from both fractions independently and the fractions and immunoprecipitates were assayed for CagA and CagF content by immunoblotting [Western blotting (WB)]. (b) Membrane preparations and soluble fractions from the 26695 wild-type strain (wt) and its isogenic *cagA* (ΔA) and *cagF* (ΔF) mutants were compared by immunoblotting with respect to membrane association of CagA and CagF.

after recombinant expression or during infection of phagocytic cells (Moese *et al.*, 2001). The fractions with the highest concentrations of CagA also contained most of the CagF (Fig. 5b). Thus, CagF shifts from a molecular mass of 35 or 70 kDa, when produced alone, to a complex of 180–250 kDa when CagA is present. Although the resolution of our gel filtration experiments was too low to determine the exact stoichiometry, this finding is consistent with the formation of complexes containing one full-length CagA monomer (135 kDa) and two CagF (35 kDa) monomers.

CagF interacts with a C-terminal CagA domain distinct from the putative translocation signal

To delineate the CagA region that interacts with CagF, we used a GST pulldown assay with recombinant GST–CagF fusion protein coupled to glutathione Sepharose beads. We incubated these beads with extracts of *H. pylori* mutants producing GSK-tagged full-length CagA or GSK-tagged

Fig. 5. Analytical gel filtration chromatography of purified recombinant CagF and a complex of CagA and GSK–CagF. (a) Purified CagF was obtained by thrombin cleavage of GST–CagF bound to glutathione Sepharose and applied to a Superdex 75 gel filtration column. Starting with the exclusion volume, fractions were collected and assayed for CagF content by immunoblotting and by densitometry. Arrows with molecular masses indicate the elution volumes of marker proteins. (b) A complex of CagA and GSK–CagF was purified by affinity chromatography on glutathione 4B from a lysate of an *E. coli* strain coexpressing *gst-cagA* and *gsk-cagF* and subsequent thrombin cleavage. The purified complex was applied to a Superdex 200 gel filtration column, and fractions were collected starting from the exclusion volume, and assayed for CagA and CagF content by immunoblotting and densitometry. Arrows with molecular masses indicate the elution volumes of marker proteins.

truncation variants (Fig. 6a). All variants were soluble as determined by immunoblotting of high-speed centrifugation supernatants of the corresponding bacterial lysates (data not shown). Immunoblots of the pulldown fractions using an anti-GSK antiserum showed that GST–CagF bound to GSK-tagged full-length CagA, whereas GST alone did not bind (Fig. 6b). All CagA variants containing their C-terminal parts were also pulled down by GST–CagF, whereas no interaction with CagA variants containing only

Fig. 6. Delineation of the CagF-interacting region of the CagA protein. (a) Schematic representation of GSK-tagged and untagged CagA truncation variants used for GST–CagF pulldown assays. The number of amino acids deleted from the N or C terminus of CagA is indicated. (b) Cell extracts from *H. pylori* strains producing the respective CagA truncation variants were subjected to GST pulldown (PD) assays with GST–CagF and GST. Control immunoblots show the presence of comparable amounts of the CagA variants in the cell extracts. GSK or CagA immunoblots [Western blots (WB)] of the pulldown fractions obtained with GST–CagF or with GST alone indicate specific interactions of CagA variants with CagF.

N-terminal regions occurred (Fig. 6b). Notably, the CagA variant lacking 347 C-terminal amino acids, which corresponds to the major 100 kDa breakdown product, was not pulled down, showing that CagF binds only to the C-terminal region of CagA, with the C-terminal 195 amino

acids being sufficient for the interaction. Since the C-terminal CagA region contains an essential part of the translocation signal (Hohlfeld *et al.*, 2006), one explanation would be that CagF acts as the main signal recognition protein. To test this possibility, we also performed GST–

CagF pulldown assays with strains producing a GSK-tagged CagA variant lacking its 20 C-terminal amino acids, or an untagged CagA variant lacking 92 C-terminal amino acids, both of which are translocation-defective (Hohlfeld *et al.*, 2006). Interestingly, both CagA variants were able to interact with GST–CagF in the pulldown assay (Fig. 6b), indicating that the CagF-binding domain is located between amino acids 1019 and 1123 of CagA. These data also suggest that CagF interaction with CagA is not sufficient for CagA recognition as a type IV substrate and that CagF binding is thus not the basic type IV signal recognition mechanism.

The CagF-binding region is required for a dominant-negative effect of GFP–CagA fusion proteins

Some secretion chaperones in type III secretion systems are thought to contribute to the translocation signals of their cognate effector proteins, targeting the effector proteins to the type III secretion apparatus (Cheng & Schneewind, 1999; Lee & Galan, 2004). The close proximity of the CagF-binding region to the C-terminal CagA signal region might indicate a similar role for CagF in type IV secretion apparatus targeting. We have previously shown that a fusion of GFP to the C-terminal half of CagA (GFP–CagA-601C) exerts a dominant-negative effect on wild-type CagA translocation (Hohlfeld *et al.*, 2006). This phenotype was interpreted as an obstruction of the translocation channel by the rigid tertiary structure of GFP, which would mean that the CagA C-terminal domain contains a recruitment signal to the type IV secretion apparatus. In order to demonstrate a potential targeting function of the CagF-binding domain, we constructed a fusion of GFP to the C-terminal 195 amino acids of CagA, produced this fusion in a wild-type *H. pylori* background (strain P1), and examined translocation of wild-type CagA to AGS cells by measuring tyrosine phosphorylation. Intriguingly, the GFP–CagA-195C fusion interfered with wild-type CagA translocation in a fashion similar to the GFP–CagA-601C fusion protein (Fig. 7). In contrast, fusion proteins consisting of GFP and the C-terminal 50 or 100 amino acids of CagA, or GFP alone, did not interfere with wild-type CagA translocation, when produced in strain P1. These results suggest that the CagF-binding domain, together with the C-terminal CagA signal region, contains sufficient information to recruit CagA to the secretion apparatus.

DISCUSSION

Translocation of CagA evokes pronounced changes in host cell functions, such as an inactivation of Src-family kinases accompanied by protein dephosphorylation (Odenbreit *et al.*, 2000; Selbach *et al.*, 2003, 2004), a disturbance of epithelial tight junction integrity (Amieva *et al.*, 2003), and massive rearrangements of the actin cytoskeleton that are

Fig. 7. Dominant-negative effects of GFP fusions to C-terminal CagA regions. Gene fusions of *gfp* to *cagA* portions encoding the indicated numbers of C-terminal amino acids, or *gfp* alone, were expressed in *H. pylori* wild-type (wt) strain P1. After standard infection of AGS cells, wild-type CagA translocation from the corresponding strains was determined by tyrosine phosphorylation. A CagA immunoblot [Western blot (WB)] of the same fractions indicates the presence of equal amounts of CagA, and a GFP immunoblot shows the expression of the corresponding GFP–CagA fusion proteins.

apparent in cell culture as the so-called hummingbird phenotype or an induction of cell motility (Segal *et al.*, 1999; Churin *et al.*, 2003; Mimuro *et al.*, 2002; Higashi *et al.*, 2002, 2004; Suzuki *et al.*, 2005). Moreover, host cells respond to CagA injection with altered signal transduction activities and modified gene expression patterns (Guillemin *et al.*, 2002; Umehara *et al.*, 2003; El Etr *et al.*, 2004; Franco *et al.*, 2005; Yokoyama *et al.*, 2005). Accordingly, CagA injection into host cells has important consequences for host–bacterium interaction and would thus be expected to be subject to strict control. In line with this assumption is the observation that recognition of CagA as a substrate for type IV secretion seems to be more complex than recognition of other type IV substrates (Hohlfeld *et al.*, 2006). Four proteins encoded on the *cag* PAI (CagF, CagI, CagZ and Cag β) are necessary for CagA translocation without being secretion apparatus components (Fischer *et al.*, 2001b). The identification of CagF as a CagA-interacting protein (Couturier *et al.*, 2006) and its characterization as a chaperone-like molecule support the notion of a quality control process prior to CagA translocation.

Secretion chaperones and chaperone-like molecules are involved in different types of protein secretion, but have been extensively characterized in type III secretion systems. In these systems, their function is to maintain the stability and secretion competence of effector proteins, e.g. by keeping them in an unfolded state, to prevent premature

effector protein interactions and/or to recruit the effector proteins to the secretion apparatus (Parsot *et al.*, 2003; Ghosh, 2004). In many cases, however, effector protein translocation is not strictly dependent on the presence of the secretion chaperone. For type IV secretion systems, a secretion chaperone function has been documented for the VirE1 protein of *A. tumefaciens*, which is necessary for translocation of the effector protein VirE2 into plant cells (Sundberg *et al.*, 1996; Deng *et al.*, 1999; Zhou & Christie, 1999; Sundberg & Ream, 1999; Zhao *et al.*, 2001). The IcmS, IcmW and LvgA proteins of the *L. pneumophila* Icm/Dot system, which are required for translocation of a subset of substrates and have also been termed 'type IV adaptor proteins' (Ninio *et al.*, 2005; Bardill *et al.*, 2005; Vincent & Vogel, 2006), probably have a similar function. Several lines of evidence argue for a function of CagF as a secretion chaperone-like molecule for CagA:

(i) Like VirE1 and the *L. pneumophila* type IV adaptor proteins, CagF has typical features that are also common to type III secretion chaperones: it has an acidic isoelectric point (pI 4.5) and a high predicted content of α -helical domains. However, with a size of 35 kDa, it is much larger than typical secretion chaperones, which usually have a molecular mass of less than 20 kDa. This unusual size may relate to the membrane-association properties of CagF.

(ii) CagF is necessary for CagA translocation, but it is not required for IL-8 induction, which is thought to occur independently of CagA translocation, possibly by leakage of peptidoglycan fragments through the type IV secretion apparatus (Viala *et al.*, 2004). In the case of *H. pylori* P12, which belongs to a subset of strains in which CagA has an ancillary IL-8-inducing function (Brandt *et al.*, 2005), CagF is required only for the CagA-dependent fraction of the IL-8-inducing effect.

(iii) Like most secretion chaperones in type III secretion systems, CagF forms homodimers, and our gel filtration data are consistent with the formation of a 2:1 stoichiometry in the complex with CagA. The calculated size of a complex of one full-length CagA monomer and two CagF monomers would be 205 kDa, which corresponds quite well with the elution volume of the peak fractions. Since the CagA Δ 347C variant does not interact with CagF, it is unlikely that the 100 kDa CagA breakdown product found in these fractions participates in the CagA–CagF complexes. Secretion chaperones in type III secretion systems are known to also bind in a 2:1 chaperone:effector protein ratio via hydrophobic patches on the chaperone surface and hydrogen bonds (Ghosh, 2004). For type IV secretion systems, the oligomeric status of chaperone-like molecules is less clear. VirE1 binds to VirE2 with a 2:1 stoichiometry, although VirE1 itself seems to form tetramers rather than dimers (Zhao *et al.*, 2001). In contrast, IcmS seems to form two different heterodimeric complexes with either IcmW or LvgA, which together may also form tetramers (Ninio *et al.*, 2005; Vincent & Vogel, 2006).

(iv) The CagF-binding domain of the CagA protein is, together with the C-terminal secretion-signal-containing region, sufficient for the dominant-negative effect induced by GFP fusions. Since such dominant-negative effects do not occur with epitope tags such as the GSK tag fused to the same CagA regions (data not shown) they are unlikely to be caused simply by competition for CagF binding. Instead, they suggest a recruitment function for the C-terminal CagA region and thus a contribution of CagF to the targeting signal, as also suggested for some type III secretion chaperones (Cheng & Schneewind 1999; Lee & Galan, 2004).

Unlike typical secretion chaperones, CagF seems to be present in both soluble and membrane-associated pools. Although a clear separation of *H. pylori* membranes is not easily achieved by standard techniques, such as differential detergent extraction or sucrose-density-gradient centrifugation (Exner *et al.*, 1995; Fischer *et al.*, 2001a), our fractionation experiments indicate that CagF is most likely associated with the cytoplasmic membrane, as already suggested elsewhere (Couturier *et al.*, 2006). The fluorescence of the CagF–GFP fusion and the extraction of CagF with 1 M NaCl suggest that CagF is peripherally associated with the cytoplasmic face of the inner membrane. The fact that CagF interacts with CagA primarily at the cytoplasmic membrane, and only to a very limited extent in the cytoplasm, indicates that the mechanism of action of CagF is very different from that of VirE1. VirE1 is a cytosolic protein that functions by regulating efficient translation of VirE2 (Zhao *et al.*, 2001) and by preventing the formation of VirE2 aggregates (Deng *et al.*, 1999; Zhao *et al.*, 2001). In contrast, CagF does not seem to have a role in stabilizing CagA or preventing it from aggregation, since both the formation of higher order oligomers or aggregates and the processing of recombinant CagA produced in *E. coli* were independent of the presence or absence of CagF (data not shown). Furthermore, CagF is not necessary for CagA recruitment to the membrane, which is probably a prerequisite for type IV translocation. CagA membrane recruitment is also independent of any other *cag*-encoded factor, since its distribution between membrane-associated and soluble states was unchanged in the mutant P12 Δ PAI[pJP55], which expresses *cagA* in a *cag* PAI-deficient background (data not shown). However, CagA interaction with the membrane might not be sufficient to target the secretion apparatus. The location of the site of assembly of the Cag secretion apparatus is currently unknown, although extracellular type IV-associated surface appendages are often found in a polar localization or along the lateral surface of bacteria (Rohde *et al.*, 2003). The localization of the CagF–GFP fusion to the bacterial cell is reminiscent of the distribution of the Cag secretion apparatus components CagY and CagT over the bacterial surface (Rohde *et al.*, 2003). Recently, an interaction between CagF and the cytoplasmic domain of CagY has been described (Busler *et al.*, 2006), supporting the assumption that CagF is located in proximity to the Cag

type IV secretion system. Thus, we speculate that interaction with CagF at the cytoplasmic membrane is a prerequisite for targeting CagA to type IV secretion apparatus assembly sites. This is also supported by the dominant-negative effect observed by fusion of GFP to the CagA C terminus including the CagF-binding region. Accordingly, interaction with CagF may contribute to the CagA targeting signal, as has been suggested for type III secretion chaperone-effector complexes such as the SycE-YopE complex in *Yersinia enterocolitica* (Birtalan *et al.*, 2002). VirE1, in contrast, is not necessary for the targeting of its cognate effector protein VirE2 to the VirB/D4 secretion apparatus (Atmakuri *et al.*, 2003).

It is currently unknown which component recognizes CagA as a type IV secretion substrate. Recent investigations have shown that different type IV effector proteins have secretion signals at their C-terminal ends (Nagai *et al.*, 2005; Vergunst *et al.*, 2005; Schulein *et al.*, 2005). Although a similar sequence is present at the CagA C terminus as well, the additional requirement for the CagA N terminus indicates that CagA recognition as a substrate is more complex than in other type IV secretion systems (Hohlfeld *et al.*, 2006). Since the CagA N terminus is dispensable for the dominant-negative effect of GFP fusions, this suggests that a second binding or recognition step is necessary for translocation. Interestingly, the outer-membrane apparatus component VirB9 of *A. tumefaciens* has been reported to have such an additional substrate selectivity function (Jakubowski *et al.*, 2005); however, the VirB/D4 system accomplishes substrate translocation with only 19 C-terminal amino acids, although less efficiently (Vergunst *et al.*, 2005). The current hypothesis states that recognition of C-terminal type IV secretion signals and thus substrate specificity is mediated by the coupling proteins (Gomis-Rüth *et al.*, 2004; Christie *et al.*, 2005; Cambronne & Roy, 2006). After recognition by the coupling protein, substrates are transferred to the cognate ATPases of the VirB11 family (Cascales & Christie, 2004; Atmakuri *et al.*, 2004), which might represent the entry point into the translocation channel. Although there is no direct experimental evidence so far, the coupling protein homology of Cag β and its requirement for CagA translocation suggest a similar role for Cag β in CagA secretion signal recognition. Since CagA variants lacking 20, 54 or 92 C-terminal amino acids are translocation-incompetent but still able to interact with CagF, it is unlikely that CagF itself has a signal recognition function. The direct interaction of CagA and CagF in the absence of other CagA translocation factors or secretion apparatus components rather suggests that CagF binding is the first step in the process of CagA translocation and thus precedes recognition of the translocation signal. Nevertheless, CagF binding may increase the accessibility of the C-terminal secretion signal to the signal recognition factor or may keep the C terminus partially unfolded. It has been speculated that a disordered state of the C terminus is important for signal recognition in type IV secretion systems (Amor *et al.*, 2005). Since we could not detect

GSK-CagF phosphorylation after AGS cell infection and since CagF did not coprecipitate with tyrosine-phosphorylated CagA from infected cells (data not shown), it is unlikely that CagF is translocated together with CagA to epithelial cells. Thus, we would expect that CagA entry into the translocation channel results in the release, and possibly recycling, of CagF.

Taken together, the results presented in this study confirm that recognition of CagA as a substrate for type IV secretion is a complex process and argue for a function of the translocation factor CagF as a CagA secretion chaperone-like molecule. They suggest that CagF binding might be a quality control mechanism that ensures the integrity or the correct folding of the CagA substrate prior to recognition of its translocation signal and entry into the translocation channel. Further studies are required to elucidate the molecular details of this critical step in *H. pylori* pathogenesis.

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