

The *Helicobacter pylori* CagA protein induces cortactin dephosphorylation and actin rearrangement by c-Src inactivation

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The gastric pathogen *Helicobacter pylori* translocates the CagA protein into epithelial cells by a type IV secretion process. Translocated CagA is tyrosine phosphorylated (CagA^{P-Tyr}) on specific EPIYA sequence repeats by Src family tyrosine kinases. Phosphorylation of CagA induces the dephosphorylation of as yet unidentified cellular proteins, rearrangements of the host cell actin cytoskeleton and cell scattering. We show here that CagA^{P-Tyr} inhibits the catalytic activity of c-Src *in vivo* and *in vitro*. c-Src inactivation leads to tyrosine dephosphorylation of the actin binding protein cortactin. Concomitantly, cortactin is specifically redistributed to actin-rich cellular protrusions. c-Src inactivation and cortactin dephosphorylation are required for rearrangements of the actin cytoskeleton. Moreover, CagA^{P-Tyr}-mediated c-Src inhibition downregulates further CagA phosphorylation through a negative feedback loop. This is the first report of a bacterial virulence factor that inhibits signalling of a eukaryotic tyrosine kinase and on a role of c-Src inactivation in host cell cytoskeletal rearrangements.

Keywords: actin cytoskeleton/molecular pathogenesis/
type IV secretion/tyrosine phosphorylation

Introduction

Type III and type IV secretion systems mediate the direct injection of virulence factors into host cells and play a key role in the pathogenesis of numerous bacteria that infect plants or animals (Finlay and Falkow, 1997; Lee and Schneewind, 2001). Type III secretion systems translocate protein effectors through a syringe-like device and have been detected in pathogens such as *Yersinia* and *Salmonella* species, *Shigella flexneri*, enteropathogenic *Escherichia coli* (EPEC) and *Pseudomonas aeruginosa* (Hueck, 1998; Kubori *et al.*, 1998; Galan and Collmer, 1999; Cornelis and van Gijsegem, 2000). Type IV secretion systems are functionally related but evolutionary distinct from type III machineries and mediate the transfer of DNA and/or proteins into the host cell cytoplasm (Burns, 1999; Christie and Vogel, 2000). The prototypic member of the latter transporter family is that of

Agrobacterium tumefaciens, consisting of assembled VirB and VirD proteins that drive the translocation of the oncogenic transfer (T)-DNA–protein complex into plant cells. Until now, only a few secreted protein effectors of type IV transporters have been described, such as pertussis toxin of *Bordetella pertussis* (Burns, 1999), DotA and RalF from *Legionella pneumophila* (Nagai and Roy, 2001; Nagai *et al.*, 2002) and CagA from the gastric pathogen *Helicobacter pylori* (Segal *et al.*, 1999; Asahi *et al.*, 2000; Backert *et al.*, 2000; Odenbreit *et al.*, 2000; Stein *et al.*, 2000).

In *H. pylori*, type I strains harbour the *cag* (cytotoxin-associated genes) pathogenicity island (*cagPAI*), whereas type II strains lack the entire *cagPAI* (Censini *et al.*, 1996; Akopyants *et al.*, 1998). Among several *H. pylori* virulence determinants, like VacA or NapA (Montecucco and Rappuoli, 2001), the *cagPAI* has raised special interest because *cagPAI* positive-isolates significantly increase the risk of infected patients developing severe gastritis, peptic ulcer disease or even gastric cancer (Peek and Blaser, 2002). Accordingly, WHO has classified *H. pylori* as a class I carcinogen (IARC, 1994). The *cagPAI* encodes homologs of a VirB/D complex known from other type IV secretion systems, the 120–150 kDa immuno-dominant antigen CagA and several other proteins with unknown functions (Censini *et al.*, 1996). It is now well established that *H. pylori* actively injects CagA into target cells in a *cagPAI*-dependent manner (Montecucco and Rappuoli, 2001). Furthermore, this *H. pylori* type IV secretion system stimulates the production of pro-inflammatory cytokines and chemokines by infected host cells in a CagA/VirD4-independent manner, possibly by translocating another as yet unknown factor or by direct activation of a cell surface receptor (Crabtree *et al.*, 1995; Censini *et al.*, 1996; Selbach *et al.*, 2002a). Systematic mutagenesis has revealed that many genes throughout the whole *cagPAI* are essential for both CagA translocation and the induction of pro-inflammatory responses (Fischer *et al.*, 2001; Selbach *et al.*, 2002a).

Upon translocation into the host cell cytosol, CagA undergoes tyrosine phosphorylation (Segal *et al.*, 1999; Asahi *et al.*, 2000; Backert *et al.*, 2000; Odenbreit *et al.*, 2000; Stein *et al.*, 2000). Phosphorylation of CagA occurs within the C-terminus of the protein and is mediated by members of the Src family of tyrosine kinases (Selbach *et al.*, 2002b; Stein *et al.*, 2002). The major phosphorylation motif is a cluster of Glu-Pro-Ile-Tyr-Ala (EPIYA) sequence repeats that share homology to c-Src consensus phosphorylation sites. The number of these EPIYA motifs varies from 1–5 repeat units depending on the individual CagA protein species (Covacci *et al.*, 1993; Selbach *et al.*, 2002b; Stein *et al.*, 2002). For example, Y972 was identified as the most important phosphorylation site in CagA from the TIGR *H. pylori* strain (Backert *et al.*, 2001).

CagA phosphorylation was found to be a prerequisite for the induction of actin cytoskeletal rearrangements in AGS gastric epithelial cells (Backert *et al.*, 2001; Stein *et al.*,

2002). The characteristic morphology of infected cells has been referred to as the 'hummingbird phenotype' (Segal *et al.*, 1999). This phenotype resembles hepatocyte growth

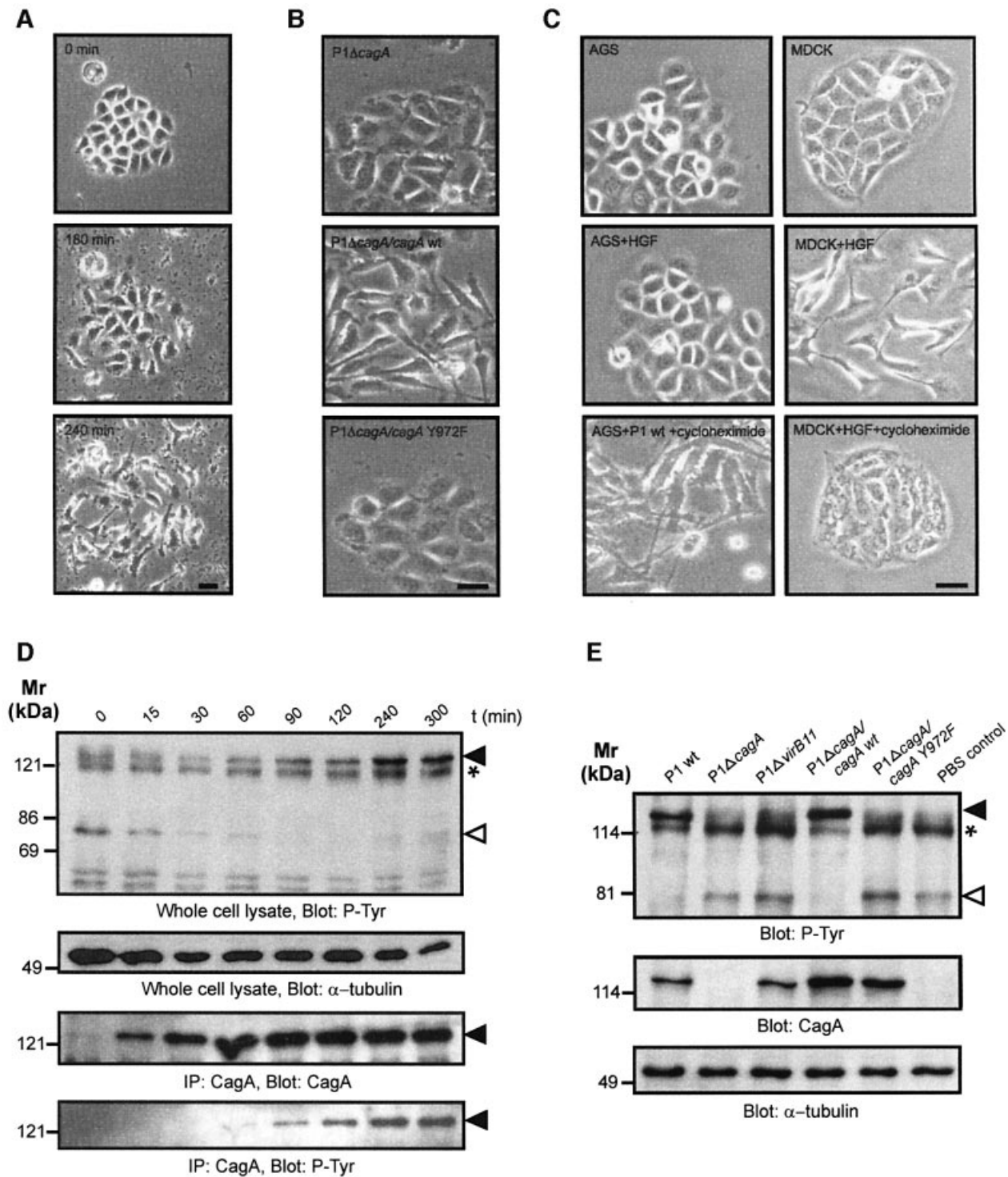


Fig. 1. *Helicobacter pylori*-induced cytoskeletal rearrangements depend on CagA tyrosine phosphorylation and are associated with dephosphorylation of p80. (A) Live cell imaging of an AGS cell cluster infected by wild-type *H. pylori* reveals cell elongation and scattering. (B) An isogenic *cagA* gene mutant does not stimulate this response. Complementation with wild-type *cagA* (P1ΔcagA/cagA) but not *cagA* lacking the tyrosine phosphorylation site (P1ΔcagA/cagA Y972F) restores the phenotype. (C) HGF (20 pM) induced scattering of MDCK but not AGS cells. Treatment of cells with cycloheximide (10 μg/ml) prevents HGF-induced MDCK cell scattering but not the *H. pylori*-induced phenotype in AGS cells. (D) The time course of CagA tyrosine phosphorylation (filled arrowhead) parallels dephosphorylation of p80 (open arrowhead). In order to distinguish CagA^{P-Tyr} from a 120 kDa host cell protein (asterisk) CagA was also immunoprecipitated (lower panels). (E) Effect of different *H. pylori* mutants on the tyrosine phosphorylation pattern of AGS cells. Dephosphorylation of p80 strictly correlates with CagA phosphorylation. α-tubulin blots served as loading controls. Scale bars: 20 μm.

factor (HGF)-induced scattering of Madin–Darby Canine Kidney (MDCK) cells. HGF binds to the HGF receptor c-Met and activates a signalling cascade which ultimately leads to the dissociation of epithelial cells (Weidner *et al.*, 1990; Stella and Comoglio, 1999). However, the mechanism by which *H. pylori* induces scattering of AGS cells is not understood. Recently, the protein tyrosine phosphatase (PTPase) Shp-2 was shown to bind specifically to transiently expressed CagA^{P-Tyr} via its src homology 2 (SH2) domain followed by the activation of the Shp-2 PTPase activity (Higashi *et al.*, 2002). Independent reports have demonstrated that CagA^{P-Tyr} initiates the dephosphorylation of several as yet unidentified host cell proteins (Backert *et al.*, 2000; Püls *et al.*, 2002). However, whether the latter events are linked to the activation of Shp-2 and the induction of cytoskeletal rearrangements or if actin binding proteins like the Arp2/3 (actin related protein) complex and N-WASP might play a role in this scenario remains to be clarified (Stein *et al.*, 2002).

Here we identify cortactin, an actin binding protein and c-Src substrate, to be dephosphorylated in a CagA^{P-Tyr}-dependent manner. Significantly, the subcellular location of cortactin changes upon *H. pylori* infection, implicating an important role of this protein for the CagA-mediated rearrangement of the actin cytoskeleton. Moreover, we show that phosphorylation of CagA leads to inhibition of

c-Src resulting in cortactin dephosphorylation. Since activated c-Src prevents both cortactin dephosphorylation and cytoskeletal rearrangements, these events are critically involved in CagA^{P-Tyr}-induced signalling to the host cell cytoskeleton.

Results

CagA^{P-Tyr} induces cytoskeletal rearrangements and host protein dephosphorylation

AGS gastric epithelial cells acquire an elongated shape with needle-like protrusions upon infection with wild-type *H. pylori*. We followed these changes in morphology by live cell imaging (Figure 1A). After bacterial attachment the cells lose their cell–cell contacts and start migrating. This cell scattering effect was fully established after 240 min infection with wild-type bacteria. Induction of these cytoskeletal rearrangements were not observed in cells infected with isogenic P1Δ*cagA* mutant (Figure 1B). Complementation of our *cagA* mutant with wild-type *cagA* (P1Δ*cagA/cagA*) restored the cellular phenotype (Backert *et al.*, 2001). However, *H. pylori* P1Δ*cagA* expressing *cagA* mutated at the known phosphorylation site (P1Δ*cagA/cagAY972F*) did not induce cytoskeletal rearrangements (Figure 1B, lower panel), indicating that both CagA translocation and phosphorylation at Y972 are essential.

The morphology of infected AGS cells is reminiscent of cell scattering induced by HGF receptor (c-Met) signalling. In MDCK cells, the morphogenic properties of HGF depend on gene transcription and translation (Rosen *et al.*, 1990). Thus, we sought to explore whether CagA^{P-Tyr} stimulates an HGF-like response in AGS cells. As expected, cell scattering was induced in HGF-treated MDCK control cells, however, exposure of AGS cells to 20–100 pmol HGF did not induce cell scattering (Figure 1C, middle panel). Cell scattering was blocked by addition of the protein translation inhibitor cycloheximide in HGF-stimulated MDCK cells but not in AGS cells infected with *H. pylori* (Figure 1C, lower panel). Thus, scattering of infected AGS cells does not require protein biosynthesis. Therefore, the cellular mechanisms of *H. pylori*-induced scattering of AGS cells and HGF-induced scattering of MDCK cells are considerably different.

Our findings suggested that the CagA^{P-Tyr}-induced cellular phenotype involves an early host response that is independent of *de novo* protein synthesis. In order to pinpoint early host signalling events upon accumulation of intracellular CagA^{P-Tyr}, we first investigated tyrosine phosphorylation in AGS cells infected with wild-type *H. pylori* in a time course (Figure 1D). CagA phosphorylation was determined both in whole cell lysates (upper panel) and in anti-CagA immunoprecipitates (lower panel). We found that accumulating amounts of CagA^{P-Tyr} were temporally correlated with the dephosphorylation of an ~80 kDa host cell protein p80 (upper panel, open arrowhead) upon infection with wild-type *H. pylori*. Like the cytoskeletal rearrangements, dephosphorylation of p80 required CagA phosphorylation on Y972 because the P1Δ*cagA/cagAY972F* mutant had no effect (Figure 1E). These results raised the possibility that CagA phosphorylation, dephosphorylation of p80 and rearrangements of the

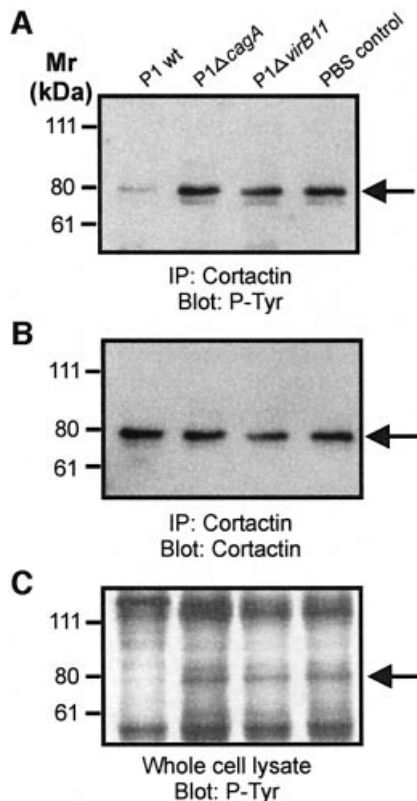


Fig. 2. Cortactin is specifically dephosphorylated upon infection with wild-type *H. pylori*. Cortactin was immunoprecipitated from infected AGS cells with a monoclonal anti-cortactin antibody. Probing with a phosphotyrosine-specific antibody reveals dephosphorylation of cortactin (A). The anti-cortactin blot shows that equal amounts of cortactin were precipitated (B). Tyrosine phosphorylation pattern of whole cell lysates are shown as control (C). Arrows indicate the position of cortactin on the gels.

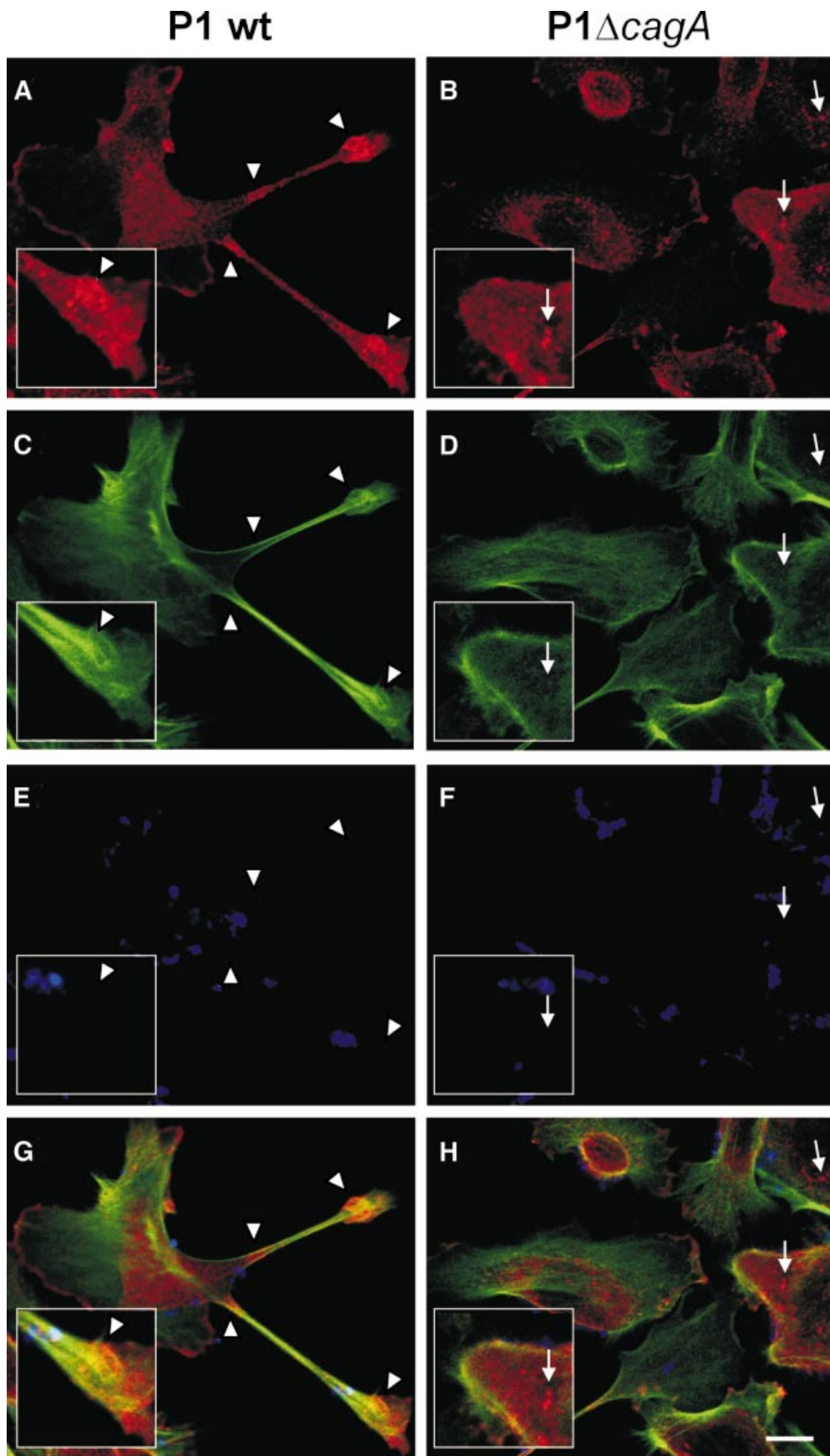


Fig. 3. Cytoskeletal rearrangements are associated with cortactin re-localization. AGS cells infected with wild-type *H. pylori* (A, C, E and G) or the isogenic *cagA* mutant (B, D, F and H) were stained with anti-cortactin antibody (A and B), phalloidin (C and D) and anti-*H. pylori* antiserum (E and F). Confocal laser scanning microscopy reveals cortactin co-localization with F-actin at the tip and the base of the protrusions (G, arrowheads). In cells infected with the *cagA* mutant, cortactin has a spot-like distribution throughout the cytoplasm (H, arrows). Scale bar: 10 μ m.

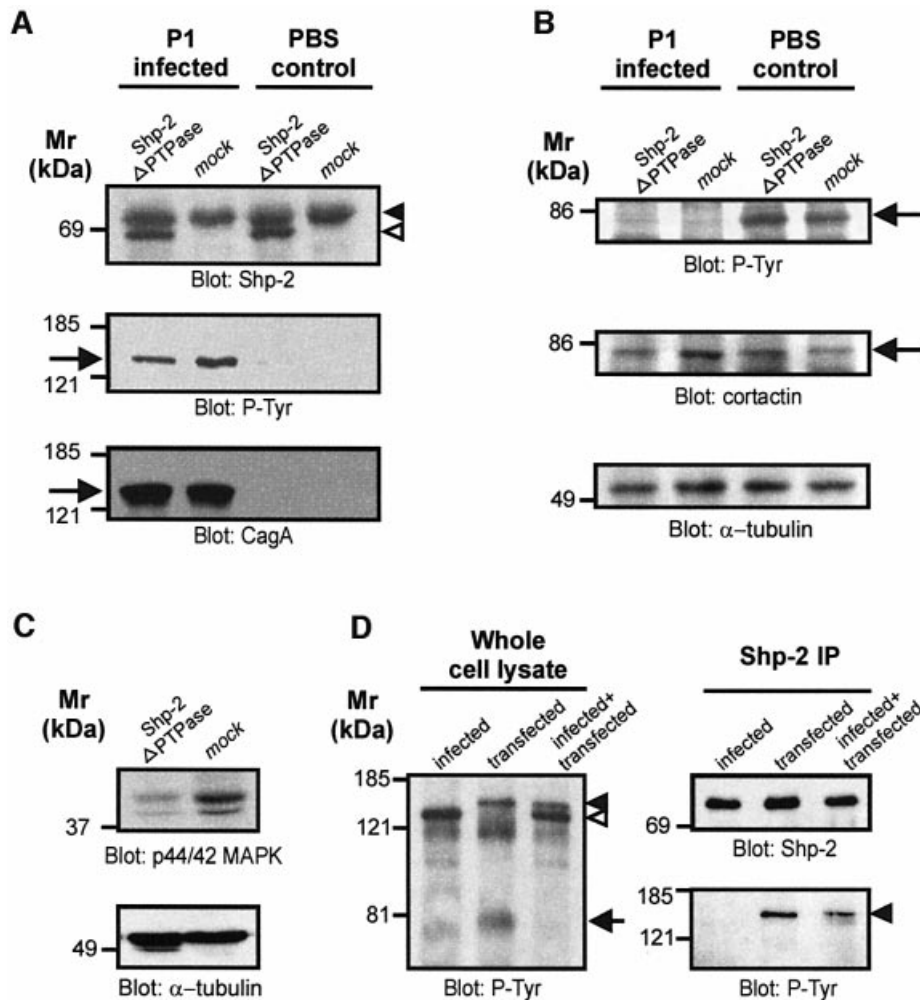


Fig. 4. Cortactin dephosphorylation is independent of the PTPase Shp-2. Cells were transfected with a *shp-2* construct deleted in the phosphatase domain (*shp-2* Δ PTPase) that acts in a dominant-negative manner. (A) Shp-2 Δ PTPase is expressed in AGS cells (upper panel, open arrowhead) and migrates below endogenous Shp-2 (filled arrowhead). Shp-2 Δ PTPase does not significantly affect CagA tyrosine phosphorylation (lower panels, arrow). (B) *Helicobacter pylori* induces cortactin dephosphorylation (arrow) irrespective of Shp-2 Δ PTPase expression. (C) As a control for dominant-negative function Shp-2 Δ PTPase-induced repression of basal MAPK activity was assayed with an activation specific antibody. α -tubulin blots served as loading controls. (D) Cells were infected with *H. pylori*, transfected with a CagA expression construct or transfected and infected simultaneously. Only CagA^{P-Tyr} translocated by live bacteria (open arrowhead), but not transfected CagA^{P-Tyr} (closed arrowhead), induced cortactin dephosphorylation (arrow). Conversely, only transfected but not translocated CagA^{P-Tyr} co-immunoprecipitated with Shp-2 (right panels).

actin cytoskeleton might be functionally related to each other.

Cortactin is the dephosphorylated host protein p80

We hypothesized that p80 dephosphorylation might be crucial for *H. pylori*-induced actin cytoskeletal rearrangements to occur and sought to identify this protein. Cortactin is an 80 kDa actin binding protein that has recently emerged as a central regulator of the actin cytoskeleton since it has been shown to stimulate the actin nucleation activity of the Arp2/3 complex (Urano *et al.*, 2001; Weaver *et al.*, 2001). To test whether cortactin is the dephosphorylated host cell protein, AGS cells were infected with wild-type *H. pylori* and *cagPAI* mutant strains for 6 h followed by immunoprecipitation of cortactin and western blotting using an anti-phosphotyrosine antibody. Indeed, wild-type *H. pylori* but not the

P1 Δ *cagA* and P1 Δ *virB11* mutants induced the dephosphorylation of cortactin (Figure 2A). As a control, reprobing of the blot with an anti-cortactin antibody revealed similar amounts of cortactin present in the immunoprecipitates (Figure 2B). Respective phosphotyrosine patterns of whole cell lysates are given for comparison (Figure 2C). Thus, CagA^{P-Tyr} induces the dephosphorylation of the actin binding protein cortactin.

Cortactin is recruited to the tips of needle-like protrusions in infected AGS cells

In order to investigate the cellular localization of cortactin in AGS cells upon infection with *H. pylori*, confocal laser scanning microscopy was applied. In AGS cells infected with wild-type bacteria, cortactin predominantly co-localized with filamentous actin (F-actin) at the tip and at the base of the characteristic cellular protrusions (Figure 3A, C and G, arrowheads). In contrast, in non-

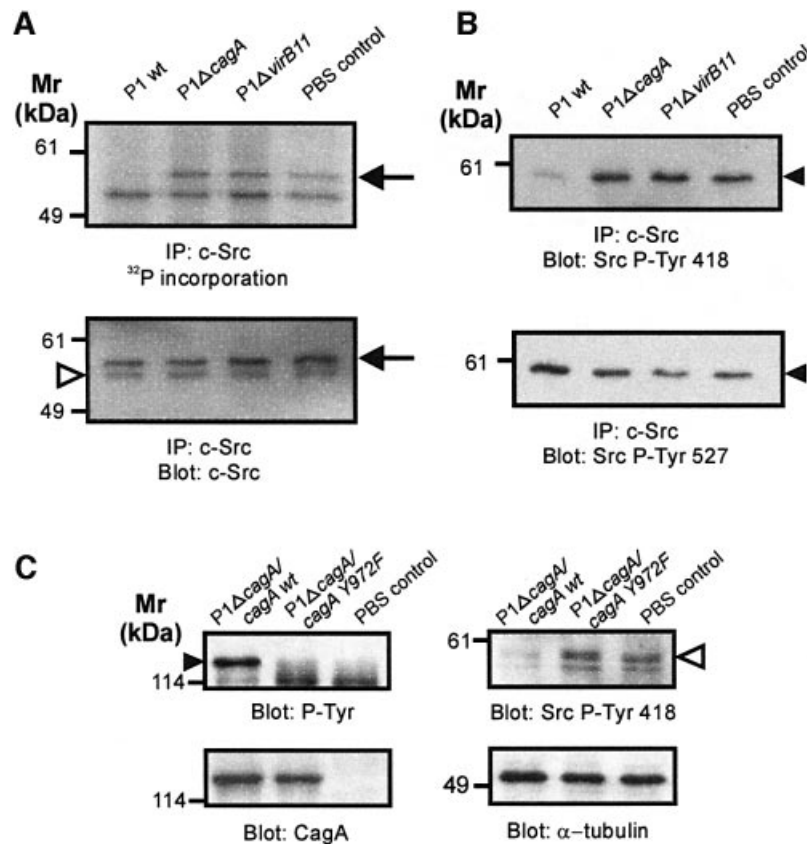


Fig. 5. CagA^{P-Tyr}-specific inactivation of c-Src. (A) The catalytic activity of immunoprecipitated c-Src was determined by an *in vitro* kinase assay with [γ -³²P]ATP. *Helicobacter pylori* wild-type infection strongly reduces c-Src autophosphorylation (upper panel, arrow). Blotting with a c-Src-specific antibody shows that similar amounts of c-Src were precipitated (lower panel, arrow). The arrowhead marks the immunoglobulin heavy chain. (B) Western blotting with phosphospecific anti-c-Src antibodies revealed that *H. pylori* induces c-Src inactivation by dephosphorylation of Y418 (upper panel) and phosphorylation of Y527 (lower panel). (C) Effect of CagA phosphorylation (filled arrowhead) on c-Src inactivation. Only bacteria complemented with wild-type *cagA* (P1Δ*cagA*/*cagA*), but not *cagA* mutated in the phosphorylation site (P1Δ*cagA*/*cagA*Y972F) induced Y418 dephosphorylation in c-Src (open arrowhead).

infected AGS cells (data not shown) or cells infected with the *cagA* mutant, cortactin was predominantly detected in a spot-like pattern which did not extensively co-localize with F-actin (Figure 3B, D and H, arrows). Thus, cortactin is significantly redistributed in AGS cells and co-localizes with F-actin upon wild-type infection.

Dephosphorylation of cortactin proceeds in a Shp-2-independent manner

Overexpressed CagA^{P-Tyr} in AGS cells has been reported to co-immunoprecipitate with Shp-2 (Higashi *et al.*, 2002). This suggested that CagA^{P-Tyr}-induced activation of Shp-2 could mediate the dephosphorylation of cortactin. In order to test whether Shp-2 is involved in the dephosphorylation of cortactin, we specifically inhibited Shp-2 by overexpression of a dominant-negative Shp-2 construct (*shp-2ΔPTPase*). This construct encodes a truncated Shp-2 protein deleted in the catalytic PTPase domain and has been shown to inhibit the activity of endogenous Shp-2 in a dominant-negative manner (Tang *et al.*, 1995). We have used this PTPase deletion construct, rather than serine substitution of the catalytic cysteine, because Cys to Ser mutants can sequester substrates (Bliska *et al.*, 1992). Moreover, transiently transfected Shp-2ΔPTPase (open arrowhead) can be readily distinguished from endogenous

Shp-2 (filled arrowhead) due to its smaller size (Figure 4A, upper panel). Importantly, we found that expression of Shp-2ΔPTPase had no significant effect on the amount of CagA^{P-Tyr} in infected samples (Figure 4A, arrow) nor on tyrosine phosphorylation of cortactin (Figure 4B, arrow). As a control for the dominant-negative function of this construct we analysed the basal levels of active p44/p42 mitogen-activated protein kinases (MAPK) with an activation-specific antibody (Cai *et al.*, 2002). In agreement with its dominant-negative function, we found that Shp-2ΔPTPase expression significantly reduced MAPK activation in AGS cells (Figure 4C). Collectively, these results indicated that CagA^{P-Tyr}-induced cortactin dephosphorylation is independent of Shp-2 catalytic activity.

In order to analyse whether translocated CagA^{P-Tyr} interacts with Shp-2 as has been reported for transfected CagA by Higashi *et al.* (2002), we compared whole cell lysates from infected and transfected cells. We were able to distinguish the two CagA species due to their different sizes (Figure 4D, left panel). While both translocated (open arrowhead) and transfected CagA (closed arrowhead) were tyrosine phosphorylated, only translocated CagA induced cortactin dephosphorylation (arrow). However, despite the higher amount of translocated CagA^{P-Tyr}, only transfected CagA^{P-Tyr} co-immunoprecipi-

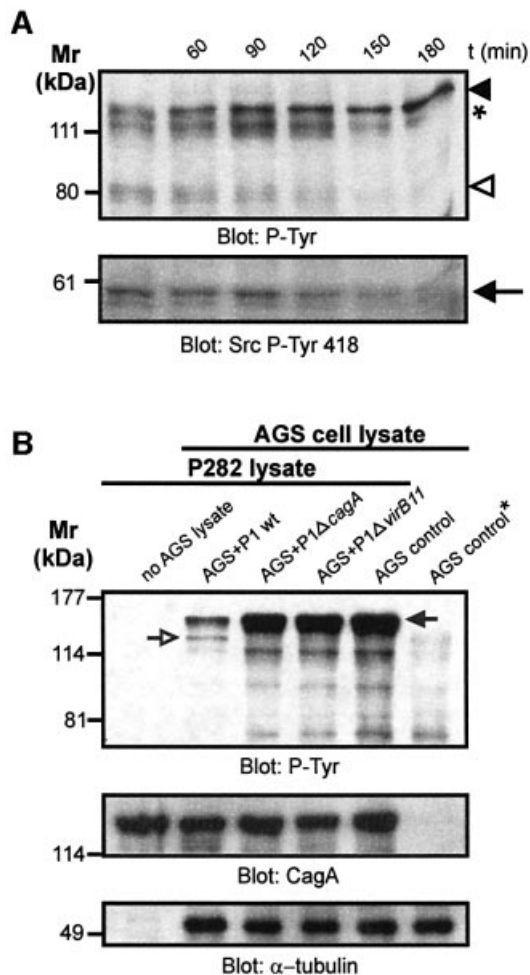


Fig. 6. CagA^{P-Tyr}-induced c-Src inactivation inhibits succeeding CagA phosphorylation. (A) A time course reveals that accumulation of CagA^{P-Tyr} (filled arrowhead) in the host cell correlates with the degree of cortactin dephosphorylation (open arrowhead) and c-Src inactivation (arrow). The asterisk denotes a 120 kDa host cell protein. (B) CagA^{P-Tyr} inhibits succeeding CagA phosphorylation. Cells infected with the P1 strains were lysed, combined with an excess of lysate derived from the *H. pylori* strain P282 and *in vitro* phosphorylation reactions were performed. Tyrosine phosphorylation of CagA from the strain P282 (filled arrow) and P1 (open arrow) was analysed. P1 wild-type infection strongly reduced phosphorylation of CagA from strain P282 (second lane). The AGS cell control marked with an asterisk was not incubated with P282 lysate (last lane). Similar amounts of CagA and host cell lysates were present in anti-CagA and anti- α -tubulin blots (lower panels).

tated with Shp-2 (right panels). These results strongly suggest that translocated CagA from the *H. pylori* strain P1 and the TIGR strain 26695 (not shown) induces cortactin dephosphorylation in a Shp-2-independent manner.

AGS cells infected with *H. pylori* revealed strongly reduced c-Src activity

Protein tyrosine phosphorylation is regulated by both protein tyrosine kinases and PTPases. We did not detect PTPase activity in immunoprecipitated CagA nor in CagA^{P-Tyr} (data not shown). Thus, we considered the possibility that *H. pylori* might induce the dephosphorylation of cortactin by inactivation of its cellular kinase. As cortactin has been reported to be a c-Src substrate (Wu

et al., 1991), we wanted to determine the catalytic activity of c-Src during infection. For this purpose, c-Src was immunoprecipitated from whole cell lysates with a c-Src-specific antibody. The catalytic activity of precipitated c-Src was determined by an *in vitro* autophosphorylation assay in the presence of [γ -³²P]ATP (Figure 5A). We found that infection with wild-type *H. pylori*, but not P1 Δ cagA and P1 Δ virB11 mutants inhibited the catalytic activity of c-Src (Figure 5A, upper panel, arrow). As a control, reprobing of the blot with a c-Src-specific antibody verified that equal amounts of c-Src were present (Figure 5A, lower panel, arrow). Thus, *H. pylori* inhibits the catalytic activity of c-Src in a CagA-dependent manner.

Phosphorylation of CagA at Y972 results in the inactivation of c-Src

c-Src contains two important tyrosine phosphorylation sites with opposing effects on protein conformation and catalytic activity (Hunter, 1987). The activity of c-Src is repressed through intramolecular interactions between its SH2 and SH3 domains with a C-terminal tyrosine residue (Y527) and the SH2-kinase linker sequence, respectively (Hubbard, 1999). Autophosphorylation occurs at Y418 and leads to the activation of the kinase, whereas phosphorylation of Y527 (numbering according to chicken c-Src) by Csk inhibits Src kinase activity (Nada *et al.*, 1991). In order to investigate the molecular mechanism of CagA-induced Src inactivation, we determined the phosphorylation status of Y418 and Y527 with phosphospecific antibodies. We found that wild-type *H. pylori* induced both dephosphorylation of Y418 and phosphorylation of Y527 (Figure 5B, arrowheads). Thus, *H. pylori*-mediated c-Src inactivation involves both dephosphorylation of the Src autophosphorylation site and phosphorylation of the inhibitory C-terminal residue Y527. In order to determine whether CagA phosphorylation at Y972 is essential for the inhibition of c-Src, we infected AGS cells with *cagA* mutants that have been complemented either with wild-type *cagA* (P1 Δ cagA/*cagA*) or with *cagA* lacking the phosphorylation site (P1 Δ cagA/*cagA*Y972F). Again, infection with P1 Δ cagA/*cagA* induced Src inactivation (Figure 5C, open arrowhead). In contrast, the CagA phosphorylation-deficient mutant P1 Δ cagA/*cagA*Y972F did not inactivate Src. Therefore, we concluded that the inactivation of c-Src is specifically induced by CagA phosphorylated on Y972.

CagA phosphorylation is regulated through c-Src inactivation via a negative feedback loop

Since c-Src is the cellular kinase of CagA (Selbach *et al.*, 2002b; Stein *et al.*, 2002) and is subsequently inactivated by CagA^{P-Tyr}, we might have detected a negative feedback loop which could explain how phosphorylation of injected CagA is regulated. In order to test this hypothesis, we analysed the temporal correlation between CagA phosphorylation, Src inactivation, and dephosphorylation of cortactin (Figure 6A). The time course shows gradually increasing amounts of injected CagA^{P-Tyr} between 1–3 h of AGS cell infection with wild-type *H. pylori* (Figure 6A, filled arrowhead). Again, cortactin was dephosphorylated over time (open arrowhead). Reprobing of the same membrane with a phosphospecific antibody recognizing

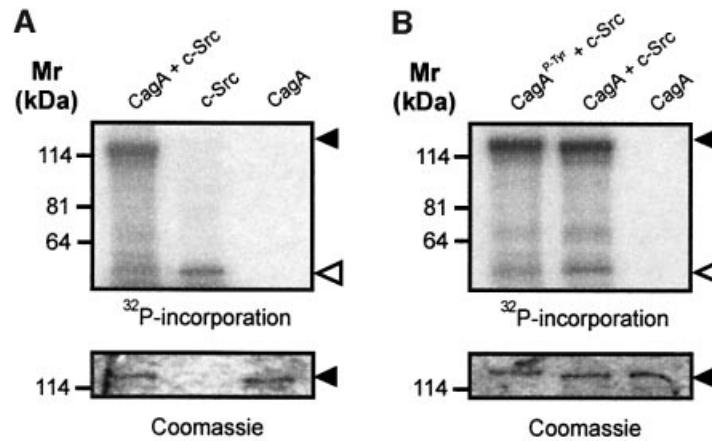


Fig. 7. Purified CagA inhibits recombinant c-Src activity *in vitro*. (A) Purified CagA significantly reduced autophosphorylation of recombinant c-Src (open arrowhead). (B) Purified CagA was phosphorylated (CagA^{P-Tyr}) with nonradioactive ATP before addition of [γ - ^{32}P]ATP (first lane). CagA^{P-Tyr} had a marked inhibitory effect on c-Src activity. The lower panels show Coomassie Blue stains of the exposed gels to ensure equal amounts of CagA were loaded.

Src phosphorylated on Y418 (active Src) revealed that the amount of active Src decreased drastically between 1–3 h after infection with wild-type *H. pylori* (Figure 6A, arrow). This suggests that there are two processes in infected AGS cells which occur in parallel: (i) CagA is phosphorylated by active Src at early stages of infection; and (ii) c-Src is gradually inactivated by the accumulation of CagA^{P-Tyr} within the host cell.

To test whether a negative feedback loop regulates CagA phosphorylation, we investigated the effect of CagA^{P-Tyr}-induced Src inhibition on the Src-mediated phosphorylation of a second CagA protein species from another *H. pylori* strain. For this purpose, AGS cells were infected with wild-type *H. pylori* strain P1, P1 Δ cagA, and P1 Δ virB11 mutants at an m.o.i. of 100. After 6 h, the infected cells were harvested, lysed and incubated with an excess of *H. pylori* lysate prepared from strain P282 in an *in vitro* phosphorylation reaction. These two *H. pylori* strains have been chosen because their CagA is phosphorylated to a similar extent during infection (data not shown) and the individual CagA protein species can be easily distinguished by their different sizes; 130 kDa (strain P1, open arrow) and 150 kDa (P282, filled arrow), respectively (Figure 6B). The results show that lysates from non-infected AGS cells or cells infected with P1 Δ cagA or P1 Δ virB11 mutants strongly phosphorylated CagA from strain P282 *in vitro*. In contrast, a markedly reduced phosphorylation of CagA from strain P282 was observed when lysate of AGS cells infected with wild-type P1 was used. Anti-CagA and anti-tubulin blots verified that identical amounts of P282 and host cell lysates were present. Thus, CagA^{P-Tyr} inhibits further CagA phosphorylation by modulating c-Src activity through a negative feedback loop.

Purified CagA inhibits c-Src *in vitro*

In order to determine whether CagA can inactivate c-Src in the absence of other cellular factors we performed *in vitro* experiments. For this purpose, we purified CagA and incubated it with recombinant c-Src. The catalytic activity of c-Src was determined by autophosphorylation. When CagA was incubated with c-Src in the presence of

[γ - ^{32}P]ATP, c-Src activity was reduced as compared with incubation of c-Src without CagA (Figure 7A, open arrowhead). Densitometric analysis revealed that this reduction was ≤ 3 -fold. Next, we wanted to know whether this inhibitory function depends on CagA phosphorylation. Therefore we tyrosine phosphorylated CagA *in vitro* by pre-incubation with c-Src and non-radioactive ATP. After 25 min, [γ - ^{32}P]ATP was added and c-Src activity was determined by autophosphorylation. We found that previously *in vitro* phosphorylation of CagA significantly enhanced its inhibitory effect on c-Src activity (Figure 7B, open arrowhead). As a control, c-Src was pre-incubated with non-radioactive ATP alone for 25 min before the CagA/[γ - ^{32}P]ATP mix was added. Coomassie Blue staining of the exposed gels shows that identical amounts of CagA were loaded. Collectively, these results demonstrate that CagA^{P-Tyr} can directly inhibit c-Src, thereby providing a direct molecular link between both proteins.

c-Src inactivation is essential for cortactin dephosphorylation

Because CagA translocation and phosphorylation resulted in both c-Src inactivation and dephosphorylation of the Src substrate cortactin, it seems plausible to assume that CagA induces cortactin dephosphorylation by inactivating c-Src. To prove this hypothesis, we transiently expressed an activated Src construct (srcY527F) in AGS cells, infected these cells with *H. pylori* and analysed cortactin tyrosine phosphorylation and the Src kinase activation status (Figure 8). Our data show that while *H. pylori* induced the dephosphorylation of cortactin in mock-transfected cells, expression of SrcY527F prevented cortactin dephosphorylation (Figure 8, upper panel, open arrowhead). *Helicobacter pylori* inhibited endogenous Src but was unable to inactivate the transiently expressed c-SrcY527F (Figure 8, second panel, arrow). Therefore, *H. pylori* induces the dephosphorylation of cortactin by inhibiting the tyrosine kinase c-Src. Interestingly, transfection of a wild-type c-Src construct did not lead to a significant increase in cortactin phosphorylation. Moreover, in AGS cells expressing the srcY527F construct, CagA became hyperphosphorylated (Figure 8, upper panel, filled

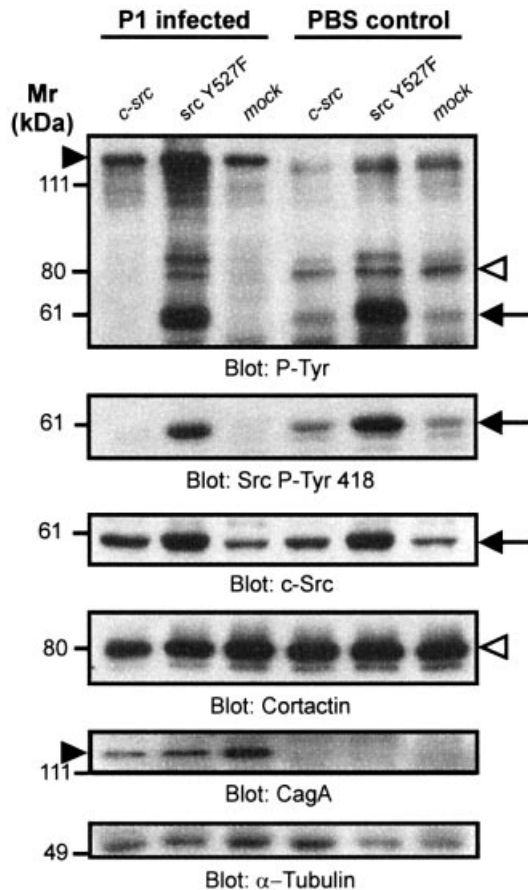


Fig. 8. CagA^{P-Tyr} induces cortactin dephosphorylation through inactivation of c-Src. AGS cells were transiently transfected with *c-src* or activated *src* (*src* Y527F) constructs. The cells were either infected with the strain P1 or left uninfected. Activated *src* (i) induced the hyperphosphorylation of CagA (filled arrowhead) and Src (arrow); (ii) prevented cortactin dephosphorylation (open arrowhead); and (iii) prevented Src inactivation (second panel, arrow). c-Src, cortactin, CagA and α -tubulin blots were performed as controls.

arrowhead), indicating that the feedback inhibition which under normal conditions regulates CagA phosphorylation was disrupted. Blots were stripped and reprobed with anti-c-Src, anti-cortactin, anti-CagA and anti-tubulin antibodies as loading controls, thereby excluding artefacts (Figure 8, lower panels).

***c-Src* inactivation is essential for cytoskeletal rearrangements**

In order to determine the role of Src inactivation and cortactin dephosphorylation for the rearrangement of the actin cytoskeleton, AGS cells were transfected with *c-src* constructs (wild-type and *src*Y527F), infected with *H.pylori* and analysed by immunofluorescence microscopy (Figure 9). For the staining procedure we chose a low concentration of the c-Src-specific antibody that did not notably stain those cells expressing only endogenous c-Src levels. In this way we were able to distinguish between non-transfected cells and cells expressing the respective construct. To ensure that all AGS cells were equally infected, wild-type *H.pylori* were centrifuged onto the cells. We found that infected AGS cells overexpressing

c-Src typically had the same elongated morphology as non-transfected control cells (Figure 9A, B and C). In contrast, infected AGS cells expressing the active Src construct had a rounded phenotype (arrow) that was clearly distinct from the elongated shape (arrowheads) of neighbouring non-transfected cells (Figure 9D, E and F). As a control, transfection of AGS cells with the *src* constructs without infection did not induce the formation of cellular protrusions (data not shown). Thus, Src inhibition by *H.pylori* is essential for rearrangement of the actin cytoskeleton.

Discussion

Direct injection of bacterial virulence factors into host cells has been described for two secretion machineries, termed type III and type IV (Finlay and Falkow, 1997; Galan and Collmer, 1999; Christie and Vogel, 2000). After translocation, these effector molecules target various components of eukaryotic signal transduction pathways in order to mediate bacterial attachment or entry, to transform the host cell or to block bacterial uptake by phagocytosis. Pathogenic *H.pylori* strains translocate CagA into gastric epithelial cells, thereby inducing cytoskeletal rearrangements that are thought to be involved in gastric disease (Montecucco and Rappuoli, 2001; Peek and Blaser, 2002). CagA has been shown to be phosphorylated on specific tyrosine residues by Src family kinases (Selbach *et al.*, 2002b; Stein *et al.*, 2002), to interact with the PTPase Shp-2 (Higashi *et al.*, 2002), and to induce dephosphorylation of as yet unidentified host proteins (Backert *et al.*, 2000; Püls *et al.*, 2002). Here we report that CagA targets eukaryotic tyrosine kinase signalling to induce cytoskeletal rearrangements. To our knowledge, this is the first report of a bacterial virulence factor that specifically inhibits tyrosine kinase signalling.

The model depicted in Figure 10 summarizes the experimental data of this study. Once *H.pylori* has attached to gastric epithelial cells, CagA is translocated into the host cell cytosol by the type IV transporter encoded in the *cagPAI* (Segal *et al.*, 1999; Asahi *et al.*, 2000; Backert *et al.*, 2000; Odenbreit *et al.*, 2000; Stein *et al.*, 2000). In this study, we show that phosphorylation of CagA at Y972 results in a marked inactivation of Src activity by a mechanism that involves both c-Src dephosphorylation at Y418 and phosphorylation at Y527. Phosphorylation of Y527 creates a binding site for the Src SH2 domain which stabilizes intramolecular interactions within the Src molecule that lead to a closed and catalytically inactive conformation (Hunter, 1987; Hubbard, 1999). Importantly, Src inactivation results in the dephosphorylation and redistribution of the actin binding protein cortactin. As the cytoskeletal rearrangements of AGS cells are independent of *de novo* host cell protein synthesis, c-Src inactivation, but not nuclear signalling, controls AGS cell scattering by modulating the organization of the actin cytoskeleton.

Interestingly, Src plays two separate roles in CagA-induced host cell signalling. First, c-Src is the kinase of CagA and activates the CagA molecule by phosphorylation of the EPIYA sequence motif. Secondly, phosphorylated CagA induces defined phosphorylation and dephosphorylation events within the c-Src molecule which

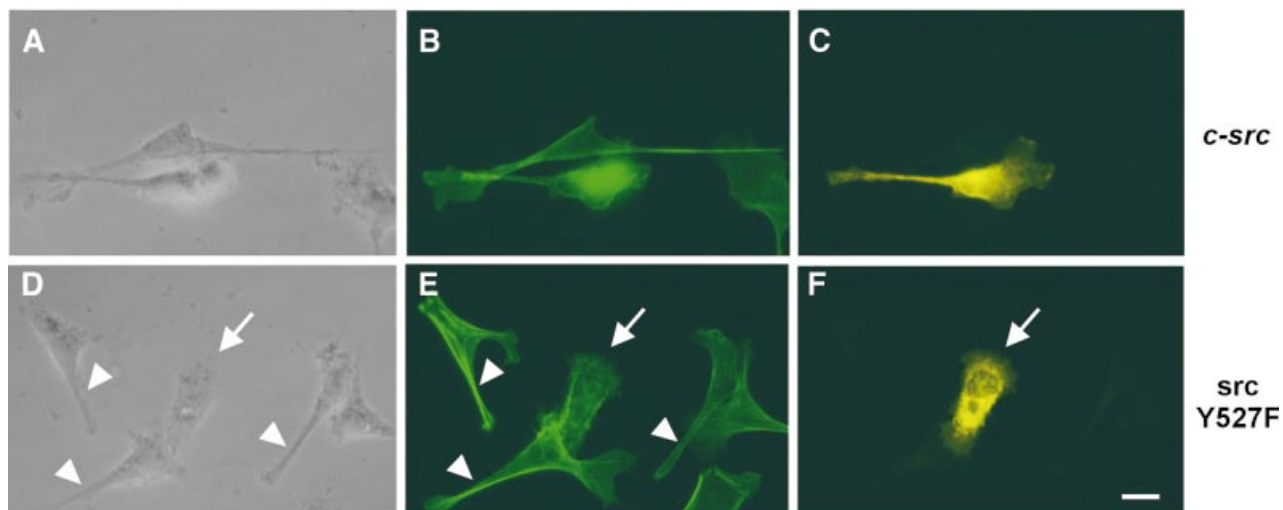


Fig. 9. c-Src inactivation is essential for cytoskeletal rearrangements. AGS cells were transiently transfected with wild-type c-Src or constitutively active SrcY527F. Subsequently, the cells were infected with *H.pylori*, stained for F-actin (B and E) and Src expression (C and F) followed by epifluorescence microscopy. While cells expressing c-Src showed characteristic actin-rich protrusions (A, B and C), constitutively active Src prevented these *H.pylori*-induced cytoskeletal rearrangements (D, E and F, arrow). Arrowheads indicate neighbouring non-transfected cells as internal control. Scale bar: 10 μ m.

lead to the inactivation of c-Src. Both processes are distinct from each other because non-phosphorylated CagA is the substrate for c-Src while only CagA^{P-Tyr} induces c-Src inactivation. Both processes together constitute a classical negative feedback loop, which provides an explanation for how the level of CagA^{P-Tyr} accumulation within the host cell is regulated. Since CagA phosphorylation appears to be a prerequisite for its pathogenic effects, this feedback loop presumably plays an important role for the control of CagA cytotoxicity.

The molecular mechanism underlying CagA^{P-Tyr}-dependent inhibition of c-Src remains unclear. CagA has no significant sequence homology to any known protein. A direct involvement of the C-terminal Src kinase Csk appears unlikely, as dominant-negative Csk had no apparent effect on CagA^{P-Tyr} function (our unpublished data). However, CagA^{P-Tyr} could possibly interact with SH2 domain-containing proteins. Furthermore, CagA contains a PxxP motif which constitutes a potential binding site for SH3 domains (Backert *et al.*, 2001). Src family tyrosine kinases contain both a SH2 and a SH3 domain (Hunter, 1987). However, stable association of CagA^{P-Tyr} with the Src SH2 and/or SH3 domains seems unlikely because this would stabilize the open conformation and therefore activate Src (Xu *et al.*, 1999). On the other hand, our observation that purified CagA can inhibit recombinant c-Src *in vitro* suggests that there is a direct interaction between both proteins. We suggest that the interaction between both proteins is transient and therefore difficult to investigate with conventional methods like co-immunoprecipitation. We propose that CagA^{P-Tyr} associates with c-Src and shifts the equilibrium from the open conformation of the kinase to the closed and inactive form. In the living cell, this inactive conformation is subsequently stabilized through phosphorylation of the negative regulation site in c-Src (Y527) by the C-terminal Src kinase Csk (Nada *et al.*, 1991) or through intermolecular autophosphorylation of the same site (Osusky *et al.*, 1995).

It would be interesting to reveal the crystal structure of CagA^{P-Tyr} in order to assess the structural basis of the interaction with c-Src.

It has been reported previously that overexpressed CagA^{P-Tyr} interacts with the PTPase Shp-2 (Higashi *et al.*, 2002). Here we demonstrate that translocated CagA^{P-Tyr} induces Src inactivation and cortactin dephosphorylation in a Shp-2-independent manner. The reason for the discrepancy between these observations is not clear. Possibly CagA derived from the strain NCTC11637 used by Higashi *et al.* (2002) has different properties compared with CagA from the strain P1 used in this study. Alternatively, translation of transfected CagA in the eukaryotic cytoplasm could effect post-translational modification, conformation or subcellular localization of the protein. It is therefore unclear whether transfected CagA and injected CagA interact with the same host cell proteins. In any case, we observed that translocated CagA from the strains P1 and 26695 (TIGR strain) can induce cytoskeletal rearrangements although it does not co-precipitate with Shp-2. This indicates that Shp-2-independent mechanisms can also induce phenotypical changes. Interestingly, another group has recently reported a Shp-2-independent interaction of CagA from a different *H.pylori* strain with the adaptor protein Grb-2 (Mimuro *et al.*, 2002). In contrast to c-Src inactivation, however, Grb-2 binding is independent of CagA tyrosine phosphorylation. Thus, CagA may have multiple functions and interfere with different eukaryotic signalling pathways.

The phenotype of *H.pylori*-infected AGS cells resembles cell scattering observed for HGF-treated MDCK cells (Weidner *et al.*, 1990; Stella and Comoglio, 1999). Consequently, it has been suggested that CagA might interfere with or mimic HGF-induced intracellular signalling (Segal *et al.*, 1999; Backert *et al.*, 2001). In this context, it is interesting to note that cortactin co-immunoprecipitates with the HGF receptor c-Met and associates with c-Met *in vitro* (Crostellina *et al.*, 2001).

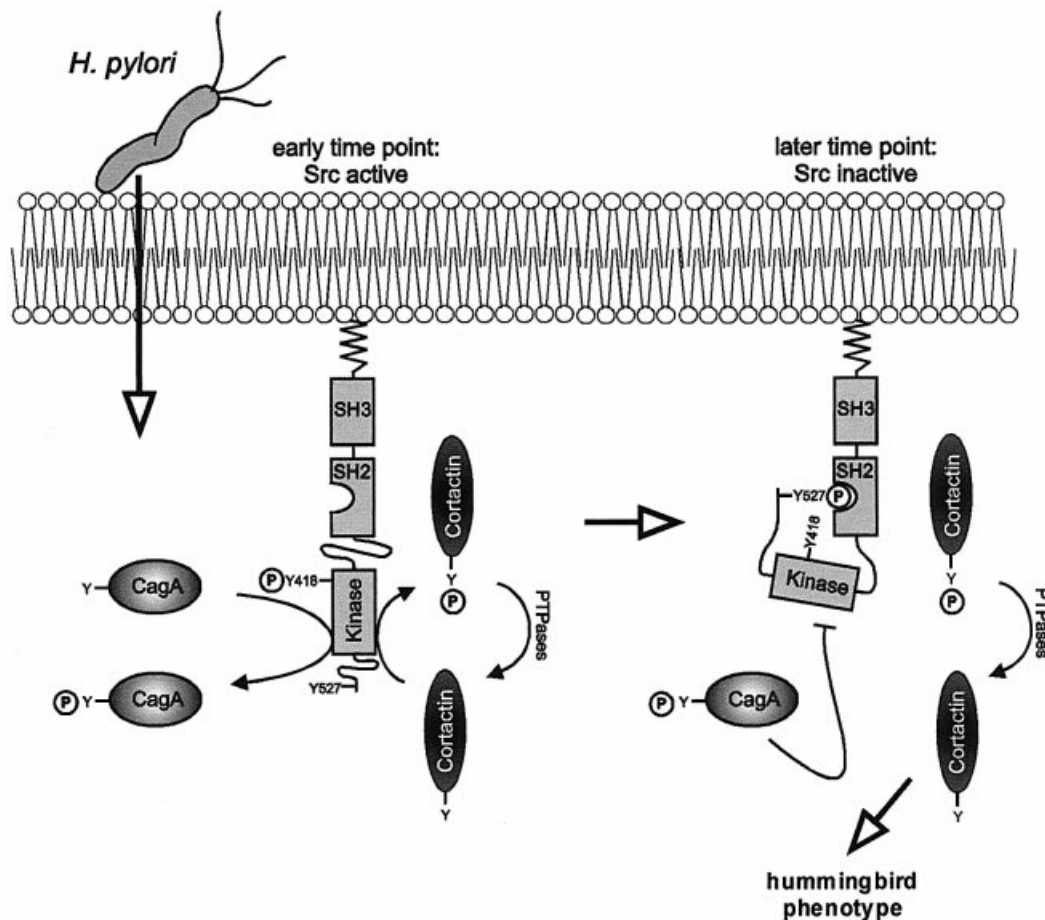


Fig. 10. Model for CagA-induced signalling leading to the cytoskeletal rearrangements of gastric epithelial cells. *Helicobacter pylori* translocates CagA by a type IV secretion dependent process. CagA is tyrosine-phosphorylated by Src which also phosphorylates cortactin. CagA^{P-Tyr} inactivates c-Src by a mechanism involving phosphorylation of Y527 and dephosphorylation of Y418. Src inactivation prevents succeeding CagA phosphorylation and leads to cortactin dephosphorylation. Dephosphorylated cortactin has enhanced actin cross-linking and/or nucleation activity and may induce the characteristic rearrangements of the actin cytoskeleton involved in cell scattering, designated as the hummingbird phenotype.

Perhaps even more relevant, Src has been shown to be required for HGF-induced cell motility (Cutrupi *et al.*, 2000). However, we demonstrate here that cycloheximide, an inhibitor of eukaryotic protein translation and HGF-induced cell scattering of MDCK cells, did not abolish the *H. pylori*-induced cytoskeletal rearrangements within AGS cells. This suggests that AGS cell scattering involves an early host response that is independent of nuclear signalling events and *de novo* protein biosynthesis.

Apart from regulating the level of CagA phosphorylation, *H. pylori*-induced Src inactivation leads to a specific dephosphorylation of the Src substrate cortactin. Several other bacterial pathogens have been reported to modify cortactin phosphorylation and/or localization. For example, invasion of epithelial cells by *Shigella flexneri* induces the tyrosine phosphorylation of cortactin by a c-Src-mediated signalling pathway (Dehio *et al.*, 1995). Similarly, invasion of endothelial cells by *Neisseria meningitidis* stimulates cortactin phosphorylation in an ErbB2/Src-dependent manner (Hoffmann *et al.*, 2001). On the other hand, enteropathogenic and enterohemorrhagic *Escherichia coli* (EPEC and EHEC, respectively) recruit cortactin to the bacterial adhesion site without apparent changes of its tyrosine phosphorylation (Cantarelli *et al.*,

2000). Consequently, phosphorylation of cortactin seems to play a role in bacterial invasion (*Neisseria* and *Shigella*) while cortactin recruitment without tyrosine phosphorylation is a common feature during attachment of extracellular bacteria (EPEC and EHEC). To our knowledge, *H. pylori* is the first known pathogen that specifically induces the dephosphorylation of cortactin. Similarly to *Shigella* and *Neisseria*, the activity of Src in infected cells determines the level of cortactin phosphorylation. In contrast to the latter pathogens, however, cortactin does not appear to be involved in *H. pylori* invasion or attachment. Although *H. pylori* can specifically enter host cells, this bacterium is a major extracellular pathogen with only few intracellular bacteria observed (Su *et al.*, 1999; Kwok *et al.*, 2002). We suggest that *H. pylori*-induced dephosphorylation and re-localization of cortactin may orchestrate more global rearrangements of the actin cytoskeleton within the entire host cell associated with AGS cell scattering.

Cortactin binds F-actin and localizes to the sites of dynamic actin assembly (Weed *et al.*, 2000), but the mechanism by which cortactin tyrosine phosphorylation and dephosphorylation events modulate the architecture of the actin cytoskeleton is unknown. Dephosphorylation

of cortactin has been reported to increase its actin crosslinking activity *in vitro* (Huang *et al.*, 1997). Therefore, dephosphorylated cortactin could cross-link actin filaments into bundles found within the characteristic cellular extensions. In addition, cortactin has been shown to activate actin polymerization by the Arp2/3 complex (Urano *et al.*, 2001; Weaver *et al.*, 2001). It will be interesting to determine whether tyrosine phosphorylation/dephosphorylation of cortactin plays a role in this process. However, CagA^{P-Tyr}-mediated inactivation of c-Src may also affect cytoskeletal rearrangements through cortactin-independent mechanism(s). For example, Src activity is required for the turnover of focal adhesions during cell migration (Fincham and Frame, 1998). Delayed focal adhesion disassembly could lead to the formation of F-actin-containing protrusions that remain attached to the extracellular matrix at the trailing edge of moving cells. Therefore, the combined effects of CagA^{P-Tyr}-mediated c-Src inactivation and *H.pylori*-induced motogenic responses (Churin *et al.*, 2001) can explain the formation of the cellular phenotype.

In summary, the characteristic changes in morphology of infected AGS cells resemble the process of oncogenic transformation. Since *cagA*-positive *H.pylori* are associated with the onset of gastric cancer (Montecucco and Rappuoli, 2001; Peek and Blaser, 2002), it is tempting to speculate that CagA^{P-Tyr} contributes to oncogenic transformation of infected cells by interfering with c-Src signalling to cortactin. Interestingly, the gene encoding cortactin is amplified in some human cancers and cortactin is suspected to play a major role in tumour invasion (Schuuring *et al.*, 1993; Patel *et al.*, 1998). It will be interesting to find out how CagA^{P-Tyr}-mediated disruption of Src signalling to cortactin could contribute to the onset of gastric cancer *in vivo*.

Materials and methods

Helicobacter pylori strains and cell culture

The generation of isogenic *H.pylori* P1 mutants and *cagA* complementation constructs has been described previously (Backert *et al.*, 2001). The *H.pylori* strain P282 was isolated from a duodenal ulcer patient. *Helicobacter pylori* were cultivated on horse serum agar plates under microaerophilic conditions by standard procedures. AGS and MDCK cells were cultivated in RPMI 1640 medium (Invitrogen) supplemented with 25 mM HEPES buffer and 10% fetal bovine serum (Gibco) for 2 days to reach monolayers of ~70% confluence. For microscopic studies, cells were seeded on acid-washed glass coverslips.

Synchronized infection assays

Helicobacter pylori were suspended in PBS and added to AGS cells at an m.o.i. of 100. Bacteria were centrifuged onto the cells for 5 min at 800 g at room temperature. After incubation at 37°C in a 5% CO₂/95% air incubator for 5–12 h, cells were washed once with PBS and harvested in ice-cold PBS containing 1 mM Na₃VO₄ (Sigma-Aldrich) and pelleted together with attached bacteria at 800 g for 5 min at 4°C.

Transfection of AGS cells

Cells were transiently transfected in 12- or 6-well tissue culture dishes using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Transfection rates were >70% as determined by co-transfection of a GFP construct. Dominant-negative Shp-2 with a deletion in the catalytic PTPase domain (Shp-2ΔPTPase) was expressed from the vector pEBB (Oh *et al.*, 1999). The construct was a gift of B.Neel. Murine c-Src and constitutively active Src in the vector pNeoMSV (Broome and Hunter, 1996) was provided by T.Hunter. Haemagglutinin-tagged CagA from the strain NCTC11637 in the vector pSP65SRα was given to us by

M.Hatakeyama (Higashi *et al.*, 2002). Transfected cells were incubated at 37°C and 5% CO₂ for 24–48 h before infection.

Immunoprecipitation

1 × 10⁷ AGS cells were lysed in Ripa buffer [25 mM HEPES, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 125 mM NaCl, 1 mM EDTA, 1 mM Na₃VO₄, Complete protease inhibitors (Roche)] by 20 passages through a 20-gauche syringe. Insoluble material was removed by centrifugation at 12 000 g for 10 min. Lysates were pre-cleared with protein G–Sepharose (Pharmacia) for 30 min at 4°C. Two micrograms of monoclonal anti-cortactin or anti-Src (Upstate) antibodies were added to the supernatants and incubated overnight. Immune complexes were precipitated by the addition of protein G–Sepharose for 1 h and washed four times in Ripa buffer. Precipitates were either used for *in vitro* kinase assays or analysed by SDS–PAGE.

In vitro phosphorylation assays

In order to determine the c-Src activity in infected AGS cells, c-Src immunoprecipitates were washed once in kinase buffer [25 mM HEPES pH 7.0, 150 mM NaCl, 10 mM MgCl₂, 1% NP-40, 5 mM DTT, 1 mM Na₃VO₄, Complete protease inhibitors] and resuspended in 40 μl kinase buffer with 10 μCi [γ-³²P]ATP (Pharmacia). Reactions were incubated at 30°C for 30 min and stopped by the addition of reducing sample buffer and boiling. c-Src autophosphorylation was detected by SDS–PAGE and autoradiography. In order to determine the effect of *H.pylori* infection of AGS cells on further phosphorylation of CagA, 1 × 10⁷ infected AGS cells (m.o.i. of 100) were lysed in 1 ml of ice-cold kinase buffer by 20 passages through a 20-gauche syringe. In parallel, 4 × 10⁹ *H.pylori* cells from the strain P282 were resuspended in 2 ml of kinase buffer and lysed in the same manner. Forty microlitres of AGS cell lysates were combined with 60 μl of *H.pylori* lysate and 40 μM ATP was added. Reactions were incubated for 5 min at 30°C and analysed by SDS–PAGE and immunoblotting.

CagA purification

For analysis of direct interaction with c-Src *in vitro*, CagA was extracted from a 2 l liquid broth culture (OD = 0.8) of the *H.pylori* TIGR strain 26695 with 8 M Urea/20 mM Tris–HCl pH 8.0 and purified under denaturing conditions by column chromatography on an anion exchange resin (Source Q15, Pharmacia) followed by gel filtration on a Superose12 HR10/30 column in the presence of 8 M Urea, 150 mM NaCl, 20 mM Tris–HCl pH 8.0. Renaturation was performed by dialysis against kinase buffer (25 mM HEPES pH 7.0, 150 mM NaCl, 10 mM MgCl₂, 1% NP-40, 5 mM DTT). Reactions with recombinant human c-Src (Upstate) and [γ-³²P]ATP were performed as described above.

Immunoblotting

SDS–PAGE, protein transfer to PVDF membranes and probing with primary and secondary antibodies was performed by standard procedures. We used monoclonal antibodies against phosphotyrosine (Santa Cruz), c-Src (Upstate), cortactin (Upstate), Shp-2 (Santa Cruz), α-tubulin (Sigma) and phospho-p44/42 MAPK (Cell Signaling). Rabbit polyclonal phosphospecific antisera against phosphorylated tyrosines 418 or 527 of Src were obtained from Biosource. CagA was detected with a polyclonal rabbit anti-CagA antibody (Schützendeller Biochemica, Tübingen, Germany). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Amersham) were used and detected with the Renaissance western blot kit system for ECL immunostaining (ICN).

Microscopy

Immunostaining was performed using standard procedures. Polyclonal anti-*H.pylori* antiserum was obtained from Biomed. Filamentous actin was detected with Alexa 488-conjugated phalloidin (MoBiTec). All secondary antibodies were purchased from Jackson ImmunoResearch. Samples were analysed by confocal laser scanning microscopy using a Leica TCS SP microscope equipped with an argon/krypton mixed gas laser source (Leica) or by epifluorescence microscopy with a Leica DM/R microscope.

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After completing the review process of this manuscript, Tsutsumi and co-workers [Tsutsumi,R., Higashi,H., Higuchi,M., Okada,M. and Hatakeyama,M. (2002) Attenuation of *Helicobacter pylori* CagA-SHP-2 signaling by interaction between CagA and C-terminal Src kinase. *J. Biol. Chem.* (epub ahead of print)] reported that CagA is capable of interacting with C-terminal Src kinase (Csk) when both proteins were overexpressed from constructs. They further showed that transfection of CagA stimulated Csk, which in turn inactivated Src kinase. Although we observed a direct inhibition of Src by CagA^{P-Tyr} *in vitro*, activation of Csk could nicely explain our finding that Src is phosphorylated on Y527. However, activation of Csk during *H.pylori* infection needs to be demonstrated in future experiments.