

SHP2-Independent Tyrosine Dephosphorylation of Cortactin and Vinculin during Infection with *Helicobacter pylori*

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The gastric pathogen *Helicobacter pylori* colonizes approximately half of the human world population. The bacterium injects the effector protein cytotoxin associated gene A (CagA) via a type-IV secretion system into host epithelial cells, where the protein becomes phosphorylated at specific EPIYA-motifs by cellular kinases. Inside the host cell, CagA can interact with over 25 different proteins in both phosphorylation-dependent and phosphorylation-independent manners, resulting in manipulation of host-cell signaling pathways. During the course of an *H. pylori* infection, certain host-cell proteins undergo tyrosine dephosphorylation in a CagA-dependent manner, including the actin-binding proteins cortactin and vinculin. A predominant response of intracellular CagA is the binding and activation of tyrosine phosphatase, the human Src-homology-region-2-domain-containing-phosphatase-2 (SHP2). Here, we considered the possibility that activated SHP2 might be responsible for the dephosphorylation of cortactin and vinculin. To investigate this, phosphatase inhibitor studies were performed. Additionally, a complete knockout mutant of SHP2 in AGS cells was created by CRISPR/Cas9 technology, and these cells were infected with *H. pylori*. However, neither the presence of an inhibitor nor the inactivation of SHP2 prevented the dephosphorylation of cortactin and vinculin upon CagA delivery. Tyrosine dephosphorylation of these proteins is therefore independent of SHP2 and instead must be caused by another, as yet unidentified, protein tyrosine phosphatase.

Keywords: Abl, Src, CagA, cortactin, vinculin, type IV secretion, T4SS, SHP2, SHP1, NSC87877

Introduction

Discovered in 1984, the gastric bacterium *Helicobacter pylori* has long been known as one of the most successful human pathogens that has infected approximately half of the global population [1]. Although most cases of infection with *H. pylori* remain asymptomatic, the bacterium has been associated with various diseases of the stomach, ranging from mild or severe gastric inflammation to peptic ulcers, and the development of gastric adenocarcinoma [2]. *H. pylori* colonizes the human stomach persistently following initial exposure that usually occurs during early childhood. The infection is most likely transferred from parents to their children, or may occur by consumption of contaminated water [3, 4]. To survive in and colonize the human stomach, the species has developed a wide array of different mechanisms. The expression of urease creates a microenvironment in which the acidity of the stomach is decreased to tolerated levels [5]. Increased motility and recognition of pH levels are also important mechanisms used by *H. pylori* to locate and penetrate the mucus layer of the stomach. *H. pylori* will continue traveling through the mucus layer until it reaches the epithelial cell lining of the human stomach [6, 7], where it uses multiple different proteins to establish prolonged or permanent colonization. Through the usage of adhesins, the bacteria achieve intimate and lasting adherence to these cells [8, 9]. Once the bacteria have adhered, they deliver a set of proteins (e.g., toxins and effector proteins) or lipopolysaccharide (LPS) metabolites (e.g., ADP-

heptose) into the host cells, which influence the course of infection and determine the development of gastric disease [10–12].

Two well-known and highly studied virulence factors of *H. pylori* are the vacuolating cytotoxin A (VacA) and the cytotoxin associated gene A (CagA). As its name suggests, VacA induces the formation of cytoplasmic vacuoles inside epithelial cells, and furthermore, it can interact with multiple cellular proteins to induce distinct responses in the host cell [13]. Various receptors were reported for VacA, which include the epidermal growth factor, glycosphingolipids and sphingomyelin, and the receptor-tyrosine-phosphatase RPTPβ [14–16]. The phosphatase RPTPβ plays a role in proliferation, adhesion, and differentiation of the host cell, all of which are important determinants for the formation of carcinoma [17]. The presence of CagA was identified as a major indicator of virulence, so that *H. pylori* strains are typically separated into 2 categories, with highly-virulent type-1 strains expressing CagA, and low virulent type-2 strains not expressing this factor [18]. Once the bacteria have made contact with host epithelial cells, they secrete the protease HtrA, an assistant of a specific type-IV secretion system (T4SS) encoded in the *cag* pathogenicity island [19]. This protease cleaves E-cadherin located in cell-cell junctions, and this cleavage opens a route for the motile bacteria to reach the intercellular space [20, 21]. Subsequently, CagA is injected by the pilus of the T4SS through the basolateral membrane into the host cell, where it becomes tyrosine-phosphorylated at specific EPIYA-sites by members of the Abl and Src tyrosine kinase family [22–25]. Inside the cell CagA interacts in either a phosphorylation-dependent or a

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phosphorylation-independent manner with a multitude of different host-cell proteins, with resultant changes in host-cell responses [26]. In its unphosphorylated state, CagA can exhibit an influence on host-cell signaling, as was shown by the transfection of phosphorylation-resistant CagA. In this form, the protein was able to induce the activation of β -catenin, disrupting cell polarity by targeting Par1b and weakening the apical-junctional complex [27–29]. In its phosphorylated state (CagA^{PY}), the protein engages in a negative feedback loop to control Src kinase activity [30, 31] and activates the MAP kinase members Erk1/2 and other kinases [32, 33].

In addition to the mentioned phosphatase RPTP β , the human Src-homology-region-2-domain-containing-phosphatase-2 (SHP2) plays an important role during *H. pylori* infection of AGS gastric epithelial cells. This phosphatase interacts with CagA^{PY}, as was shown by co-immunoprecipitation of the 2 proteins [32, 34]. SHP2 is a member of the protein-tyrosine-phosphatase (PTP) family and consists of 2 SH2-domains and 1 PTP-domain. SH2-domains recognize and bind to phosphorylated tyrosine residues of given target proteins [35], one of which is CagA^{PY}, as was shown in AGS cells. As a result of this binding, SHP2 is activated with downstream effects on host-cell signaling pathways [32]. One substrate of activated SHP2 is the focal adhesion kinase (FAK), which as a result of its SHP-catalyzed dephosphorylation is no longer active in the presence of CagA^{PY} [34].

Infections with T4SS-positive type I *H. pylori* strains have been previously shown to cause changes in the tyrosine phosphorylation status of various host-cell proteins, including cortactin [30, 33, 36, 37] and vinculin [38]. Cortactin consists of 2 isoforms, of 80–85 kDa (called p80 and p85), and in its inactive, non-phosphorylated state, it is distributed in the cytoplasm of the cell. Once cortactin is phosphorylated it targets to specific actin-rich locations. In non-infected AGS control cells, cortactin is mostly present with its tyrosine residues being phosphorylated. In this form, it can recruit members of the Arp2/3 complex to the actin filaments and, as such, influences actin branching. Thus, phosphorylated cortactin plays important roles in endocytosis and cell motility, among other processes [39–41]. Cortactin has been recognized as one of the key factors regarding the polymerization of F-actin through its binding to the Arp2/3 complex and manipulation of the cytoskeleton in general [39, 42]. The 128 kDa protein vinculin is also mostly present in its tyrosine-phosphorylated form in AGS wild-type cells. The protein is typically found in focal adhesions that anchor cells to the extracellular matrix, thereby attaching the cells to its growth surface. Vinculin further interacts with actin filaments to form a connection between the cytoskeleton and the transmembrane protein family of integrins, which form the contact points between a cell and the extracellular matrix in focal adhesions.

Thus, both cortactin and vinculin are active in their phosphorylated form, but upon infection with *H. pylori*, both proteins become dephosphorylated at their tyrosine sites. These tyrosine-dephosphorylation events are dependent on the presence of CagA^{PY}, as infections with isogenic Δ cagA mutants and mutants lacking a functional T4SS (thus being unable to deliver CagA into the cell) did not display the typical dephosphorylation of these 2 proteins [30, 38]. However, as CagA has no enzymatic phosphatase activity itself, it remains unknown which phosphatase is actually responsible for this dephosphorylation. Here, we tested if SHP2 could be the responsible enzyme, as it clearly interacts with CagA^{PY}. To investigate this, we treated AGS cells with a well-described SHP2 inhibitor prior to infection. We also created a stable AGS Δ SHP2 knockout cell line that was infected with highly virulent *H. pylori* cells.

Materials and Methods

Cultivation of Eukaryotic Cells. The human adenocarcinoma cell line AGS (ATCC CRL-1739) was grown in RPMI 1640 media (Gibco, Darmstadt, Germany) containing 10% fetal calf serum (Gibco), 1% penicillin/streptomycin (Sigma Aldrich, Steinheim, Germany), and 0.2% normocin (InvivoGen, Toulouse, France) as previously described [43]. The growth conditions for the cells were 37 °C and 5% CO₂. All antibiotics in the medium were removed by multiple washing steps before infection with *H. pylori* [44].

Creation of AGS Δ SHP2 Knockout. For the creation of the AGS Δ SHP2 knockout cell line, the CRISPR/Cas9 system was used (Santa Cruz, Heidelberg, Germany), following an adapted protocol as recently established in our lab [45]. For this purpose, AGS wild-type cells were seeded into 6-well plates and cultivated as described above. At approximately 60% confluence, the cells were transfected with 2 μ g of each of two commercially available CRISPR/Cas9 plasmids, the knockout (KO) plasmid SH-PTP2 CRISPR/Cas9 (sc-400260; Santa Cruz), and the homology-directed-repair (HDR) plasmid SH-PTP2 (sc-400260-HDR; Santa Cruz) using GeneJammer transfection reagent (Agilent Technologies, Frankfurt, Germany). The KO plasmid is able to disrupt the expression of SHP2 by inducing a double-strand-break into a 5' constitutive exon of the gene. The HDR plasmid recognizes this double strand break and repairs it, while inserting a gene for resistance against puromycin, as well as a red fluorescence protein (RFP) cassette for visual confirmation of transfection. Forty-eight-hour post-transfection, the cells were washed 2 times with Dulbeccos Phosphate Buffered Saline (DPBS, Sigma Aldrich) and given fresh RPMI 1640 with FCS, penicillin/streptomycin, and normocin as specified above, and additionally 5 μ g/mL puromycin. This concentration of the antibiotic was sufficient to kill all untransfected cells within 24 h. The thus-selected transfected cells were cultivated under these conditions to approximately 80% confluence, before a single cell was sorted into each well of a 96-well plate via fluorescence-activated cell sorting (FACS Aria II SORP; BD Bioscience). Transfection of these single cells was confirmed using their RFP signal in the mCherry channel. Of these 96 selected cells, 43 formed cell clusters within 1 week. Single-cell colonies were cultivated to approximately 70% confluency and then transferred into 48-well plates. This procedure was repeated for 24-, 12-, and 6-well plates. The single-cell colonies were cultured with RPMI 1640 media containing puromycin as described above. The complete knockout of SHP2 was confirmed by PCR and Western blotting in 9 of these colonies, while the remaining colonies only showed a knockdown of SHP2. Three of the SHP2 knockout colonies were used for further experiments with similar results.

Cultivation of *H. pylori* Strains. The T4SS-positive *H. pylori* strains NCTC11637 [1], P12 [46], and G27 [47] were grown from stocks stored at –80 °C (BHI medium containing 20% glycerol). The bacteria were grown on GC agar plates containing 10% horse-serum, 10 μ g/mL vancomycin, and 4 μ g/mL amphotericin. The microaerophilic growth conditions for the bacteria were provided in a 2.5-L anaerobic jar (Oxoid, Wesel, Germany) with a CampyGen pack [48]. The bacteria were grown for 48 h and subcultured on a fresh plate for 24 h before being harvested for infection experiments [49]. The isogenic mutant NCTC11637 Δ cagA was grown on GC agar plates as above, but the plates further contained 8 μ g/mL kanamycin.

Infection of AGS Cell Lines with *H. pylori*. AGS cells were cultured in 6-well plates (Greiner Bio-One) under growth conditions as described above to a confluency of approximately 70%. The cells were washed 2 times with PBS and given fresh RPMI 1640 media before infection. *H. pylori* bacteria were grown as described above and resuspended in BHI media. The AGS cells were then infected with *H. pylori* at a multiplicity of infection (MOI) of 100 and incubated for 6 h before the cells were harvested and lysed in a hot (95 °C) 1× SDS-PAGE buffer [50].

Western Blotting and Antibodies. Proteins were detected using SDS-PAGE and Western blotting onto PVDF membranes that were probed with primary and secondary antibodies. The following antibodies were used: α -GAPDH (Santa Cruz, sc-47724), α - β -actin (BioGenex, Berlin, Germany, #28284), α -CagA (Austral Biologicals, San Ramon, CA, USA, #HPP-5003-9), α -cortactin (Merck-Millipore, Darmstadt, Germany #05-180), α -vinculin (Sigma-Aldrich, #V9131), and α -SHP2 (Santa Cruz, sc-7384). Additionally, the following phospho-specific antibodies were used to check the phosphorylation level of the proteins: α -PY99 (Santa Cruz, sc-7020), α -phospho-vinculin (PY1064; Thermo Fischer, Darmstadt, Germany, 44-1078G), and α -phospho-cortactin (PY421; Merck-Millipore, Darmstadt, Germany, #AB3852). Secondary goat antibodies could either detect primary mouse (Invitrogen, Darmstadt, Germany, #31446) or rabbit (Invitrogen, #31460) antibodies, which were coupled to horseradish-peroxidase and developed as described [51].

SHP Inhibitor Studies. The SHP1/2 inhibitor NSC87877 (Merck-Millipore, #2613) was diluted in dimethyl sulfoxide (DMSO) to 10 mM. AGS cells were grown to 70% confluency and washed twice with PBS and fresh medium as described above. Thirty minutes before the cells were infected with *H. pylori*, they were treated with NSC87877 at a final concentration of 50 μ M or with a DMSO control. After this treatment, the cells were infected as described above.

Fractionation of AGS Cells Post-Infection. For the fractionation of AGS cells post-infection into membrane and cytosolic protein fractions, cells were seeded into Petri dishes (10 cm in diameter). All of the following steps were performed on ice with pre-cooled solutions. Six-hour post-infection, the adherent cells were removed from the Petri dish by gentle scraping. The cell suspension was centrifuged (5 min; 4 °C; 5000 rpm), and the pellet was washed with PBS and centrifuged again. The cell pellet was then incubated for 5 min with saponin buffer (50 mM Tris-HCl, pH 7.5; 0.4 mM Na₃VO₄; 1 mM NaF; 0.2% Saponin; 1× cOmplete™ Protease Inhibitor Cocktail [Roche, Mannheim, Germany]). After centrifugation (5 min; 4 °C, 13000 rpm), the cytosolic protein fraction present in the supernatant [52] was retained, while the pellet (membrane fraction) was resuspended in the saponin buffer with 1% TritonX-100, heated for 2–3 s at 95 °C, and centrifuged (5 min; 4 °C; 13,000 rpm). The solubilized membrane fraction present in the supernatant was retained, as well as the insoluble proteins in the pellet. All fractions were diluted in equal volume of 2x SDS buffer prior to SDS-PAGE.

Statistics. Relative phosphorylation levels of cortactin and vinculin were determined by quantifying band intensity levels densitometrically using Image Lab Software (Version 5.0; Bio-Rad Laboratories). The strongest band on each blot was set 100%. Error bars shown in the figures indicate \pm standard deviation. Statistical significance was evaluated using one-way ANOVA, followed by Turkey's test with GraphPad Prism statistical software (version 8.0). Significance was defined by * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$.

Ethics. No experiments were performed, which required the approval of the local ethics authorities.

Results

Tyrosine Dephosphorylation of Vinculin and Cortactin Is CagA-Dependent and Is Not Prevented by an SHP Inhibitor.

Three well-characterized T4SS-positive type-1 *H. pylori* strains, NCTC11637, P12, and G27, were used to determine tyrosine dephosphorylation of vinculin and cortactin. The 3 strains produced similar results. During infection of AGS cells with *H. pylori*, the phosphorylation state of multiple proteins changed, as demonstrated by Western blots produced from cell lysates that were stained with the pan-phosphotyrosine antibody PY99 (Figure 1A). As can be seen, translocated CagA became phosphorylated (arrow) upon infection, while CagA^{PY} was absent when infection occurred with the corresponding isogenic Δ cagA deletion mutant as expected. Further, dephosphorylation of vinculin and cortactin was demonstrated, and this dephosphorylation was CagA-dependent, as it was not observed in cells infected with the Δ cagA mutant (arrows). This is in agreement with previous studies [30, 33, 36, 38]. In addition, we noted the phosphorylation of 2 other, as yet unknown cellular proteins, at about 95 kDa and 110 kDa, respectively, in the presence of CagA-positive *H. pylori* (Figure 1A, asterisks). The expression of CagA in wild-type bacteria and its absence in the mutants were confirmed with α -CagA antibodies, and α -GAPDH antibodies served as a loading control (Figure 1A, bottom). The band intensities for tyrosine-phosphorylated vinculin and tyrosine-phosphorylated cortactin were quantified by densitometry of the corresponding PY99-stained bands (Figure 1B). To test whether SHP2 was responsible for the CagA-dependent dephosphorylation, the AGS cells were treated with the SHP inhibitor NSC87877 [53] or with solvent control prior to infection with the *H. pylori* wild-type strain NCTC11637. This produced highly similar results to the experiments performed in the absence of the SHP2 inhibitor (Figure 1C/D). The α -CagA and α -GAPDH stainings were performed with the same controls in Figure 1A and showed no loading differences (Figure 1C). Thus, it appears that the tyrosine dephosphorylation of vinculin and cortactin was independent of SHP phosphatase activity, as it could not be prevented by an SHP2-specific inhibitor.

Generation of AGS Δ SHP2 Knockout Cells. To further verify these findings, we generated a full SHP2 gene knockout in AGS cells using the CRISPR/Cas9 technology. For this purpose, a commercial DNA construct was inserted into the SHP2 gene in AGS wild-type cells to prevent its expression (Santa Cruz, Heidelberg, Germany). The construct contained a resistance against the antibiotic puromycin, as well as a RFP cassette. Transfected cells were then selected in an RPMI medium containing 5 μ g/mL puromycin. Single RFP-expressing cells were sorted by FACS and eventually grown to monolayer confluency. These were tested by Western blotting with α -SHP2 antibodies and α -GAPDH as a loading control. Three independent transfected clones were tested that were completely devoid of SHP2 signals (Figure 2A). Even long time exposure failed to identify protein bands for SHP2 (data not shown). Phase contrast microscopy of these clones revealed no obvious differences in the morphology of AGS Δ SHP2 cells compared to AGS wild-type cells (Figure 2B and data not shown). Strong signals for the tyrosine-phosphorylated forms of vinculin and cortactin were also present in all 3 knockout cell clones, similar to those of wild-type AGS (Figure 2C, Figure 2D top). The amount of detected vinculin and cortactin in the knockout cell clones and the AGS wild-type cells was similar, with reference to the α -GAPDH loading control (Figure 2D,

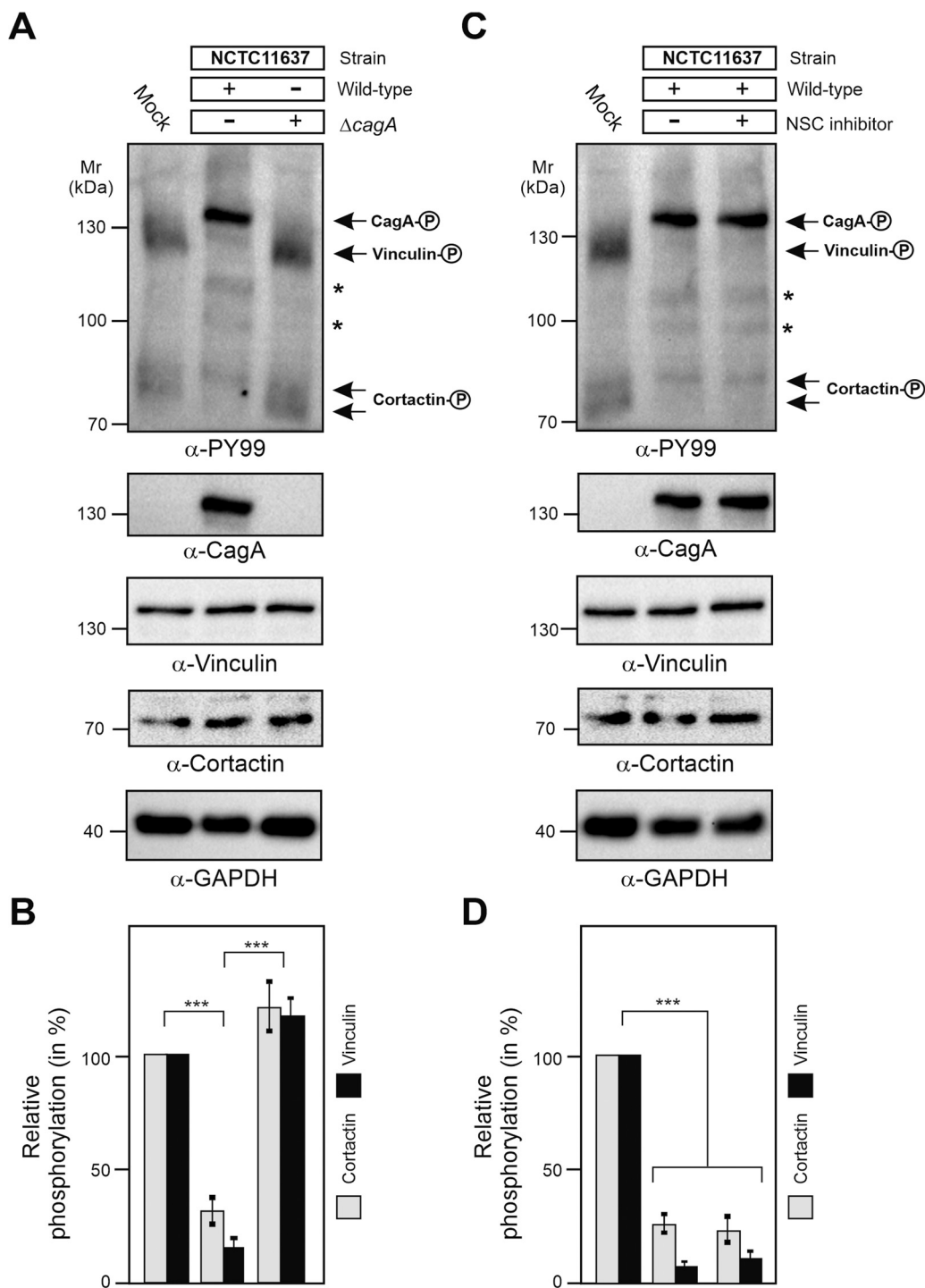


Figure 1. CagA-dependent tyrosine dephosphorylation of vinculin and cortactin is not abrogated by the SHP inhibitor NSC87877. Western blots of protein extracts from AGS cells infected with *H. pylori* NCTC11637 and a mock control are shown. Both wild-type bacteria and a $\Delta cagA$ mutant were used (A), and cells were pre-incubated with the SHP2 inhibitor prior to infection as indicated (C). Arrows show the phosphorylated forms of CagA, vinculin, and cortactin as demonstrated previously by α -PY99 antibodies [30, 38]. Two unidentified protein bands are indicated by asterisks. Loading controls were stained with α -CagA, α -vinculin, α -cortactin, and α -GAPDH antibodies. Phospho-vinculin and phospho-cortactin signal intensities were quantified by densitometric measurement of indicated bands (B, D)

bottom). These results confirm that we successfully established an SHP2 knockout in independent AGS cell clones, that such inactivation did not result in significant differences in the expression of vinculin or cortactin, and that it did not affect the PY99 phosphotyrosine signals for both proteins.

CagA-Induced Tyrosine Dephosphorylation of Vinculin and Cortactin Occurs in the Absence of SHP2 Expression. The effects of SHP2 knockout during *H. pylori* infection was investigated for all 3 strains by comparison of AGS wild-type and AGS Δ SHP2 cells. Results for strains P12 and G27 are

shown in Figure 3, with similar results obtained with strain NCTC11637 (not shown). The pan-phosphotyrosine antibody PY99 showed that infection of both AGS wild-type cells and the AGS Δ SHP2 knockout cells resulted in highly similar phosphorylation patterns. Both cell lines demonstrated the presence of phosphorylated CagA, as well as pronounced dephosphorylation of both vinculin and cortactin (Figure 3A, top). As before, 2 unknown phosphorylated proteins of approximately 95 and 110 kDa were visible, in both cell lines (Figure 3A top, asterisks). Expression of SHP2 in wild-type cells and its absence in the knockout mutant cells was

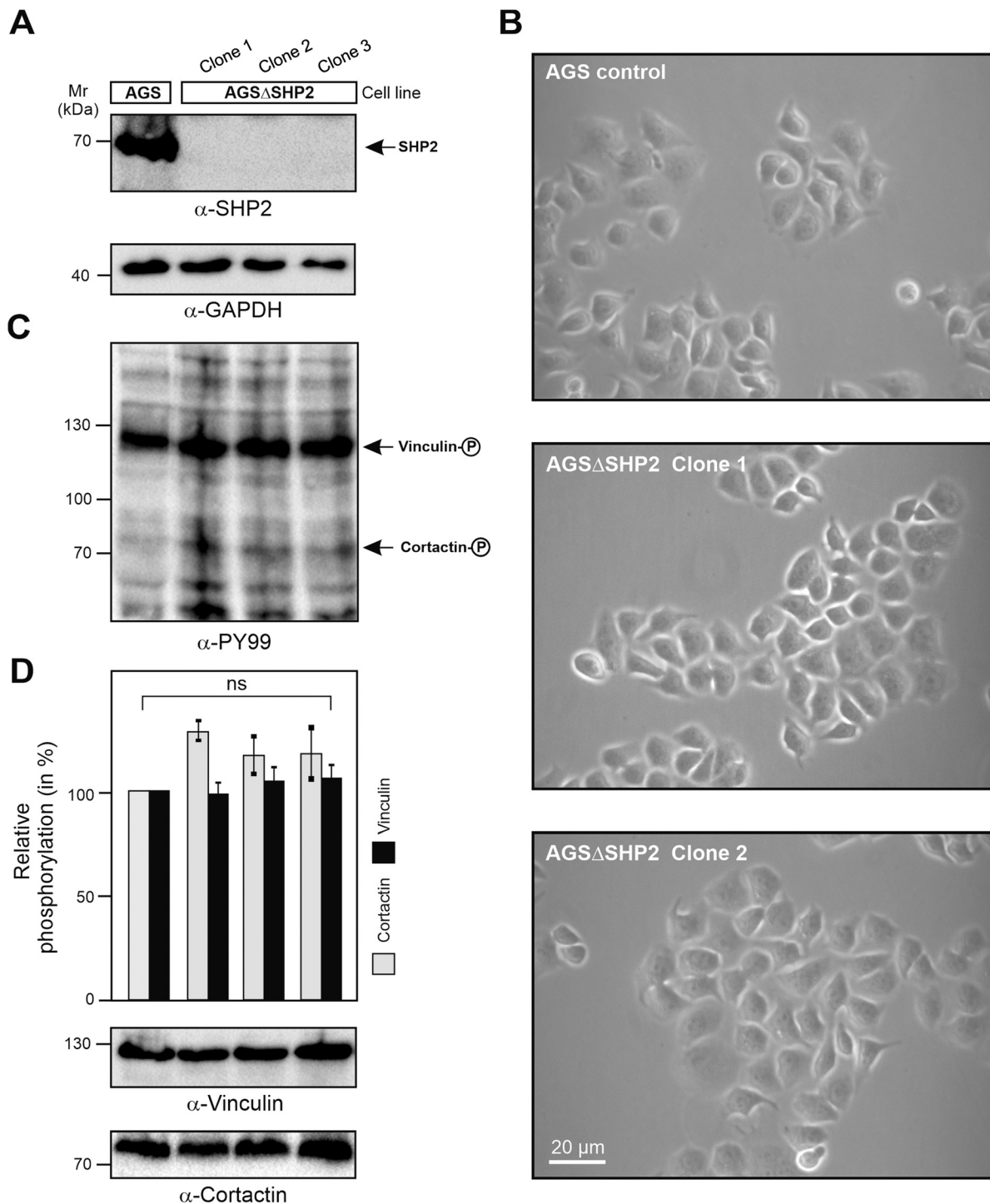


Figure 2. Generation of SHP2 knockout clones in AGS cells by CRISPR/Cas9 technology. Three knockout cell clones were completely devoid of SHP2 expression, as demonstrated with α -SHP2 antibodies (A), while levels of phosphorylated (C) and total (D) vinculin and cortactin remained unchanged compared to the wild-type control. Cell morphology was also not affected by the knockout mutation (B). Phospho-vinculin and phospho-cortactin signal intensities were quantified by densitometric measurement of indicated bands (D)

confirmed with α -SHP2 antibodies. That similar amounts of CagA were delivered into the infected cells was confirmed with α -CagA antibodies, and the α - β -actin staining confirmed that similar amounts of proteins were loaded per sample (Figure 3A, middle panels). The quantification of signals for tyrosine-phosphorylated vinculin and cortactin revealed only minor, non-significant differences between AGS wild-type and the AGS Δ SHP2 knockout cells (Figure 3A, bottom).

Tyrosine Dephosphorylation of Vinculin and Cortactin Investigated by Phospho-Specific Antibodies. To take a closer look at the dephosphorylation events of vinculin and cortactin at their specific tyrosine sites, we applied 2

commercial phospho-specific antibodies. For this, the samples used for Figure 3A were loaded onto gels again, and Western blots were now stained with an antibody specifically recognizing tyrosine phosphorylated cortactin at position 421. In agreement with the previous results, strong signals of phospho-cortactin PY421 were obtained in both uninfected mock control cell lines, while dephosphorylation at this site was clearly demonstrated following *H. pylori* infection of both wild-type AGS cells, as well as in AGS Δ SHP2 cells (Figure 3B, top). Phosphorylated vinculin was demonstrated using the phospho-vinculin PY1065 antibody. This showed that vinculin was strongly phosphorylated at tyrosine at

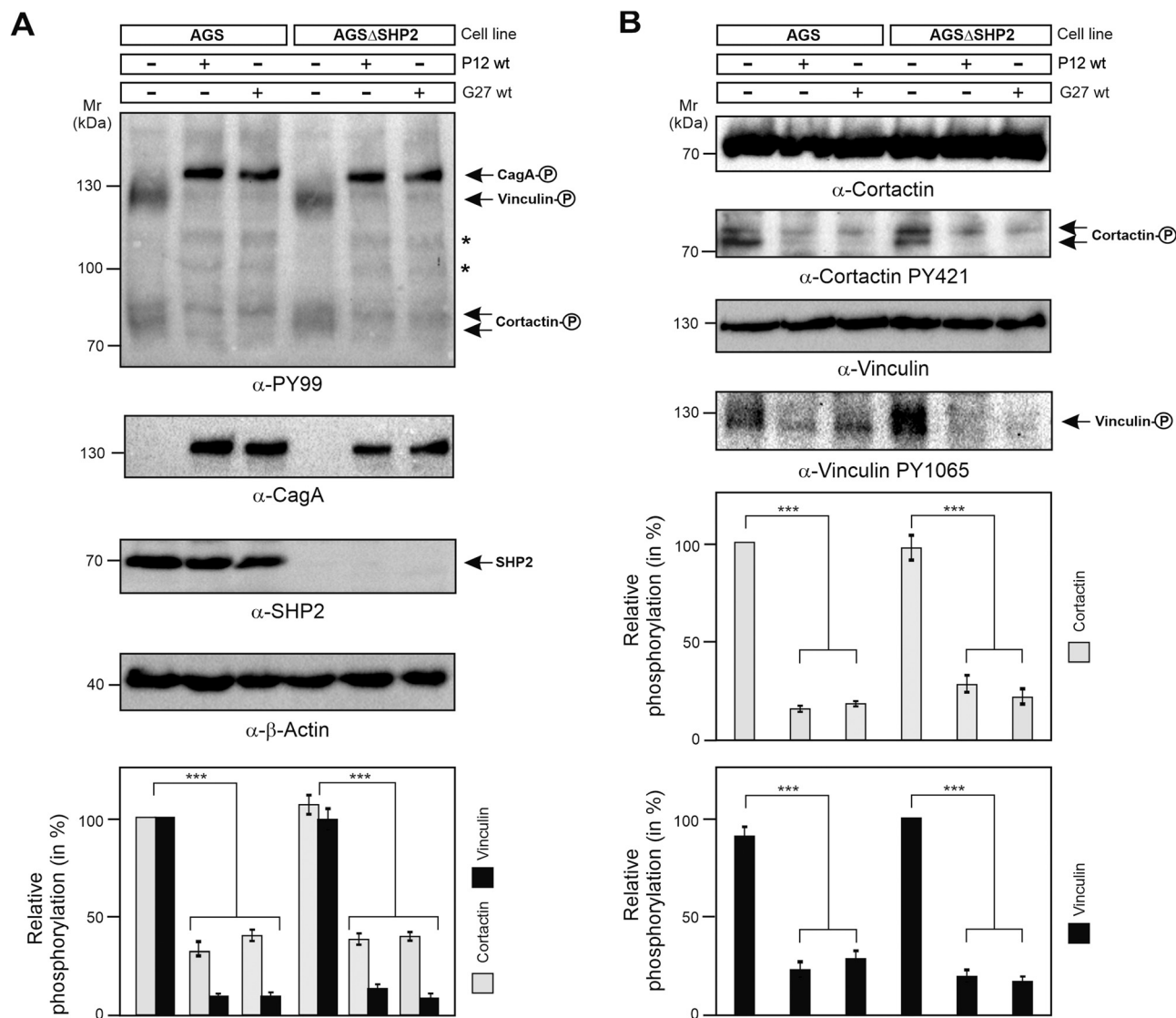


Figure 3. CagA-dependent tyrosine dephosphorylation of vinculin and cortactin by CagA does not require the expression of SHP2. The knockout cells and wild-type cells were infected with *H. pylori* strain P12 or strain G27 as indicated. Phosphorylation of cortactin and vinculin and dephosphorylation following infection were shown with α -PY99 antibodies (A) and with antibodies specific to tyrosine phosphorylation at position 421 of cortactin (PY421) and position 1065 of vinculin (PY1065) (B). This specific vinculin antibody showed no signals in protein extracts from AGS wild-type or AGS Δ SHP2 cells. Thus, a cellular fractionation approach was performed to enrich certain vinculin protein populations. Phosphorylated vinculin (PY1065) was found in the membrane fraction as expected. Phospho-vinculin and phospho-cortactin signal intensities were quantified by densitometric measurement of indicated bands and shown in the bottom graphs

position 1065 in the mock controls, but dephosphorylated after *H. pylori* infection of both wild-type AGS and AGS Δ SHP2 cells (Figure 3B, middle). The quantification of band intensities for the tyrosine-phosphorylated vinculin and cortactin (Figure 3B, bottom) confirmed that both proteins undergo tyrosine dephosphorylation at these specific phosphorylation sites upon *H. pylori* infection in an SHP2-independent fashion.

Discussion

Vinculin and cortactin are actin-binding proteins with important roles in healthy tissues. During infection of AGS cells with *H. pylori*, these proteins undergo tyrosine dephosphorylation in a CagA-dependent manner [33, 36, 38, 54]. Vinculin exhibits its primary function as an important factor in focal adhesions, thereby playing a central role in the anchoring of cells to the extracellular matrix [55]. Cortactin, on the other hand, interacts with the actin cytoskeleton and is integral to the changes in general cell shape, while it is also involved in specific signaling pathways [56]. The phosphatase SHP2 has

been proven, via immunoprecipitation and other experiments, to interact with the *H. pylori* effector protein CagA. More specifically, SHP2 is activated by the phosphorylated form CagA^{PY}, which leads to deregulated downstream host-cell signaling pathways [31, 32]. For example, SHP2 is a required component for the activation of Erk signaling pathway in response to multiple growth factors, though other growth factors (e.g., EGF) can trigger this pathway independently [57]. The phosphatase can also impact the Jak/STAT1 pathway, where cells lacking functional SHP2 exhibited much higher levels of phosphorylated STAT1 than wild-type cells upon interferon activation [58]. Furthermore, SHP2 has been shown to influence the PI3K/Akt signaling pathway and thereby promotes cell survival in fibroblasts [59]. In the present study, we investigated whether CagA^{PY} causes the dephosphorylation of vinculin and cortactin through direct engagement of activated SHP2.

Treatment of AGS wild-type cells with the SHP inhibitor NSC87877 prior to infection with *H. pylori* proved to be insufficient to prevent the dephosphorylation of either vinculin or cortactin. In fact, no difference was observed at the tyrosine

phosphorylation levels of host-cell proteins post-infection with *cagA*-positive *H. pylori* in the presence or absence of SHP inhibitor. However, a control that the inhibition had been completed was not performed. When the NSC87877 inhibitor was originally described, it was noted that SHP1 and SHP2 were inhibited at roughly the same rate, while other tyrosine phosphatases require a higher inhibitor concentration than that used here (e.g., PTP1B 5-fold, HePTP 24-fold, and DEP1 206-fold) [53]. These observations were in agreement with the results of a previous study, which had shown that the dephosphorylation of cortactin was not prevented in cells expressing a dominant-negative SHP2 construct [30]. The presence of CagA^{PY} was previously shown to cause changes in the tyrosine phosphorylation pattern in AGS wild-type cells compared to uninfected cells, which exhibited the same phosphorylation pattern as AGS wild-type cells infected with Δ *cagA* deletion mutants [30, 33, 36, 38].

Confirming evidence was obtained by the creation of AGS Δ SHP2 knockout clones, for which complete absence of the phosphatase was demonstrated. The inactivation had no effect on cell morphology, as AGS wild-type cells were morphologically identical to the AGS Δ SHP2 mutant cells. The AGS Δ SHP2 mutant cells resulted in a similar phosphorylation pattern of host-cell proteins as AGS wild-type control cells did. This held true for both uninfected cells, as well as for cells infected with various T4SS-positive *H. pylori* strains (NCTC11637, G27 and P12). When probed with phospho-specific antibodies against the phosphorylated proteins at certain tyrosine residues, the dephosphorylation of cortactin at PY421 and of vinculin at PY1065 was demonstrated.

Together, the results of the present study using NSC87877 inhibitor and CRISPR/Cas9 knockout mutants convincingly demonstrate that activated SHP2 is not the phosphatase responsible for the dephosphorylation of vinculin and cortactin. With these results, the question which tyrosine phosphatase is responsible for the dephosphorylation of vinculin and/or cortactin remains unanswered. Selbach et al. [60] analyzed the interactome of CagA^{PY} and other phosphorylated bacterial effector proteins by a proteomics-based mass spectrometry approach. CagA^{PY} was found to bind to multiple different host-cell signaling proteins through SH2 domain interaction, among which were the 2 tyrosine phosphatases SHP1 and SHP2 [60]. We consider it unlikely that SHP1 is responsible, as the inhibitor NSC87877 was described to profoundly inhibit both SHP1 and SHP2 phosphatase activities [53]. Nevertheless, the actual inhibition of either phosphatase was not experimentally confirmed here. When tyrosine dephosphorylation of vinculin and cortactin is independent of both SHP2 and SHP1 phosphatase activity, an alternative candidate host-cell tyrosine phosphatase to be considered is RPTP β . This phosphatase was also reported to be targeted by *H. pylori* and can be activated by interaction with VacA [17]. We initiated experiments using siRNA to downregulate the expression of RPTP β , and these experiments indicated that this factor is also not involved in dephosphorylation of vinculin and cortactin (unpublished data). A third alternative is the tyrosine phosphatase PTP1B. In a mouse model, cortactin was found to be a substrate of PTP1B [61], and PTP1B co-localized with vinculin in mouse fibroblasts [62]. If the same applies to human cells, this phosphatase remains a likely candidate. Studies are currently underway to investigate a potential role of PTP1B in CagA-dependent dephosphorylation of vinculin and cortactin.

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Authors' Contributions

All authors contributed to the writing of this article.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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