CORRESPONDENCE

**Helicobacter pylori** infection of AZ-521 cells reveals a type IV secretion defect and VacA-independent CagA phosphorylation

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We refer to the recent publication by Nakano and co-workers in *Disease Models & Mechanisms* (Nakano et al., 2016). This report claims that the *Helicobacter pylori* vaculating cytotoxin (VacA) is the crucial factor inducing the activation of the host cell kinase for translocated CagA, Src, via a mechanism involving the receptor phosphatase RPTP-α using the human duodenum carcinoma cell line AZ-521 as a novel model system. In fact, VacA and a type IV secretion system (T4SS) with its effectors protein CagA represent major virulence determinants of *H. pylori* (Boquet and Ricci, 2012; Foegeding et al., 2016). VacA is a paradigm of pore-forming toxins, which contribute to the pathogenesis of peptic ulceration. Several cellular receptors have been described for VacA, including various lipids, sphingomyelin, heparin sulphate, receptor protein tyrosine phosphatase (RPTP)-α, RPTP-β, EGF receptor, fibronectin and integrin β2 (CD18) on T cells. Secretion of VacA is associated with the formation of membrane channels, induction of apoptosis and inhibition of immune cell proliferation (Boquet and Ricci, 2012; Foegeding et al., 2016). The second important virulence factor is CagA, encoded by the T4SS in the cag pathogenicity island. The T4SS-pilus is induced upon host-cell contact and requires the receptor integrin α5β1 for the transport of CagA into target cells (Kwok et al., 2007). After delivery, CagA becomes tyrosine-phosphorylated (CagAPY) at EPIYA motifs by Src and Abl kinases, and mimics a host cell factor for triggering intracellular signaling cascades affecting cytoskeletal, proliferative, anti-apoptotic and other responses (Mueller et al., 2012; Backert et al., 2015). In particular, it has been demonstrated previously that the activation of Src and CagA phosphorylation proceeds in a T4SS-dependent manner (Stein et al., 2002; Selbach et al., 2003; Kwok et al., 2007), and the purified T4SS pilus-associated protein CagL alone can profoundly stimulate the activation of Src and other tyrosine kinases, via binding to integrin α5β1, in various gastric and nongastric cell lines (Tegtmeier et al., 2010).

To solve the discrepancy between the report by Nakano et al. (2016) and previous studies on the T4SS-dependent activation of Src in various cell lines (Stein et al., 2002; Selbach et al., 2003; Kwok et al., 2007; Tegtmeier et al., 2010; Mueller et al., 2012), we followed the protocol by the authors and utilized AGS and AZ-521 cell lines from the same origin as described (Nakano et al., 2016). These cells were infected with three different *H. pylori* wild-type strains and isogenic ΔvacA deletion mutants under identical conditions for 9 h at a multiplicity of infection (MOI) of 100 (Fig. 1A). The resulting protein lysates were probed with anti-PY-99 and anti-CagA antibodies to visualize the levels of CagA phosphorylation as indicative for its translocation (Kwok et al., 2007). The results show that *H. pylori* can profoundly induce CagAPY for each wild-type and ΔvacA strain in AGS cells (Fig. 1A, arrows). Surprisingly, we detected no significant difference in the CagAPY levels of wild-type strains versus those of ΔvacA mutants in AGS, and discovered no CagAPY signals at all in infected AZ-521 cells (Fig. 1A,B). This result was confirmed in at least five independent experiments including shorter and longer infection times (data not shown). Next, we infected AGS and AZ-521 cells with strain ATCC43504 as used by the authors followed by immunoprecipitation of CagA. The corresponding blots were probed with anti-CagA antibodies, confirming that equal amounts of CagA proteins were precipitated (Fig. 1C). The anti-PY-99 blot exposed for 6.1 s exhibited strong CagAPY signals in the AGS-infected samples, but not in infected AZ-521 cells (Fig. 1C). However, exposure of this anti-PY-99 blot for 77 s revealed overexpressed CagAPY signals in AGS cells and very faint bands for infected AZ-521 cells (Fig. 1D, arrow). Densitometric quantification of the signals revealed that CagA phosphorylation in AGS cells is ~163-fold to 176-fold higher than that in infected AZ-521 cells, and no significant difference was seen between the CagAPY signals from wild-type and ΔvacA mutant *H. pylori* (Fig. 1E). This suggests that either translocation of CagA or the kinase activity of Src is widely diminished in AZ-521 cells. To answer this question, we determined Src activity using an activation-specific antibody for Src phosphorylation at the autophosphorylation site Y-418. The results revealed similar strong phospho-Src signals in both cell lines (Fig. 1F). We noticed a slight reduction in overall Src activity in AZ-521 compared with AGS cells; however, this difference cannot account for the dramatic differences seen in the CagAPY signals between the two cell lines (Fig. 1G). Thus, these results strongly suggest that translocation of CagA into AZ-521 cells is widely impaired compared with that into AGS cells, rather than differences in the activity of Src. We were also unable to detect significant differences in the expression of phosphatidylinerine between the two cell lines, and propose that T4SS pilus formation or an imbalanced expression of integrin α5 and β1 chains, or lack of CEACAM receptors, could be involved in the observed T4SS defect in AZ-521 cells. This should be clarified in future studies.

As a possible explanation for the conflicting data, we assume that incubation of host cells with purified VacA and transfection of CagA as performed by Nakano and co-workers do not reflect the actual situation during infection (Nakano et al., 2016). We also assume that some observations were overinterpreted by the authors. Thus, we think that the proposal to use AZ-521 cells as a new infection model for studying novel mechanisms of type IV secretion and phosphorylation of CagA is highly questionable. Taken together, we provide evidence that AZ-521 cells exhibit a significant defect for the uptake of translocated CagA by the *H. pylori* T4SS, and therefore
Fig. 1. H. pylori infection of AZ-521 cells reveals very low type IV secretion competence compared with that of AGS cells and VacA-independent CagA phosphorylation. (A) AGS and AZ-521 epithelial cells were infected with the indicated H. pylori wild-type strains and ΔvacA mutants for 9 h using an MOI of 100 on six-well plates. Resulting protein lysates were probed with the anti-PY-99 antibody to detect CagAP[Y] as described (Mueller et al., 2012). The anti-CagA and anti-β-actin blots served as loading controls for bacterial and host cell proteins, respectively. Arrows indicate the position of CagA forms on the gels. The asterisks mark a phosphorylated 125 kDa host cell protein migrating below CagA. (B) Quantification of CagA phosphorylation signals in panel A using the luminescence image analyzer (Mueller et al., 2012). The relative CagA phosphorylation levels are shown as fold change. The signal in lane two was set as 1. Data (mean±s.e.m.) are representative of three independent experiments. (C) Immunoprecipitation of CagA from AGS and AZ-521 cells infected with H. pylori strain ATCC43504 and an isogenic ΔvacA mutant for 9 h on 10-cm petri dishes. The blots were probed with anti-PY-99 and anti-CagA antibodies and exposed for 6.1 s. CagA[Y] signals were only detected in the AGS cell samples. (D) Exposure of the anti-PY-99 blot for 77 s revealed very strong and very weak CagA[Y] signals for AGS and AZ-521 cells, respectively (arrow). (E) Quantification of CagA[Y] signals in panel D. The signal in lane five was set as 1. Data (mean±s.e.m.) are representative of three independent experiments, and show that CagA phosphorylation is more than 160-fold stronger in AGS cells than in AZ-521 cells. (F) Control blots for panels C-E showing the input levels for Src, Abl and GAPDH in lysates from the various infected cell lines. The anti-Src-PY-418 blot reveals the activation status of Src (Selbach et al., 2003; Nakano et al., 2016). (G) Quantification of anti-Src-PY-418 blot signals in F. The signal in lane one was set as 1. Data (mean±s.e.m.) indicate that Src is highly active in both AGS and AZ-521 cells.

represent no useful model system to study processes involved in CagA translocation and phosphorylation. However, although the defect of AZ-521 is remarkable and worth investigating further at the molecular level, both AGS and AZ-521 represent nonpolarized cell lines. Instead, more efforts should be directed to clarify in future how CagA can be delivered and phosphorylated in polarized gastric epithelial cell models, which are more close to the situation of H. pylori infection in vivo.

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Competing interests
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We are writing in response to the Correspondence from Tegtmeyer and Backert regarding the article by Nakano et al. (2016).

Two virulence factors, cytotoxin-associated gene A (CagA) and vacuolating cytotoxin (VacA), are required for pathological actions in *Helicobacter pylori*-infected cells (Isomoto et al., 2010; Hatakeyama, 2014). After delivery of CagA into host cells by a type IV secretion system (T4SS), it is tyrosine phosphorylated at Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs by Src and Abl family kinases. Phosphorylated CagA is responsible for subsequent biological actions in infected cells, such as modulation of signal transduction pathways (Odenbreit et al., 2000; Mueller et al., 2012). Previous studies have defined the molecular mechanism of CagA phosphorylation in host cells. CagL, a component of T4SS, a key participant in CagA phosphorylation pathways, binds to host cell protein β1 integrin, suggesting that this interaction is involved in the pathological actions of CagA in host cells (Kwok et al., 2007). Receptor protein tyrosine phosphatase alpha (RPTPα), a VacA receptor, has been shown to contribute to activation of c-Src and Src family kinases and serves as a physiological regulator of Src family kinases (Su et al., 1999).

Tegtmeyer and Backert showed in their Fig. 1A that phosphorylated CagA (pCagA) was observed in AGS cells during infection with *H. pylori*, but not in AZ-521 cells. We agree with their observations; we also observed that pCagA could not be detected in AZ-521 cells by standard western blotting using anti-pY antibody during infection with *H. pylori* (Fig. 1A). To address this observation, we performed immunoprecipitation using anti-CagA antibody to concentrate translocated pCagA proteins in AZ-521 cells. We wanted to know whether pCagA during *H. pylori* infection could be detected in AZ-521 cells. When we carried out the infection assay in AZ-521 cells by challenging with *H. pylori* strains under several experimental conditions, we could detect pCagA by infection with wild-type *H. pylori*. On the other hand, this signal could not be detected during infection with a cagPAI-deleted mutant of *H. pylori*, indicating that this signal originated with pCagA or total CagA protein (fig. 4A in Nakano et al., 2016). In addition, the presence of pCagA in AZ-521 cells during infection with *H. pylori* was verified by two additional methods (fig. 4A and B in Nakano et al., 2016). First, we examined the amount of SHP2 phosphatase by immunoprecipitation using anti-CagA antibody. Previous studies have shown that SHP2 phosphatase specifically binds to pCagA in *H. pylori*-infected host cells, suggesting that the amount of precipitated SHP2 phosphatase correlated with the amount of pCagA (Higashi et al., 2002). Second, we verified our results by examining the amount of pCagA using an antibody that recognizes phosphorylated Tyr972 in CagA, a major c-Src phosphorylation site (Backert et al., 2001; Kwok et al., 2007). Thus, these two findings support our result on the amount of pCagA. As shown in Fig. 1C, we found that Tegtmeyer and Backert also detected a corresponding pCagA signal in AZ-521 cells. Although total translocated CagA protein, precipitated with anti-CagA antibody, was similar in both AGS and AZ-521 cells, the amount of pCagA observed in AGS cells was larger than that seen in AZ-521 cells. We also showed this larger difference between AGS and AZ-521 cells, related to the phosphorylation level of translocated CagA, by immunoprecipitation using anti-CagA antibody (Fig. 1B). When we detected pCagA after immunoprecipitation on the separate membranes, pCagA in AGS cells could be detected in a very short period of time. But, detection of pCagA in AZ-521 cells required a longer period of time and a larger amount of protein than that required in AGS cells (Fig. 1C). Therefore, we think that it is difficult to evaluate the level of signal intensities of pCagA from AZ-521 cells when cell lysates derived from AGS and AZ-521 cells are loaded on the same membrane. As shown in Fig. 1D by authors of the Correspondence, signals of pCagA from AGS cells are much stronger than those seen in AZ-521 cells and, therefore, stronger signals obtained from AGS cells have an impact on the evaluation of signal intensities of pCagA obtained from AZ-521 cells. It is best to use separate gels with AGS cells and AZ-521 cells to obtain more easily validated signals.

The physiological concentration of VacA during *H. pylori* infection is not known. Therefore, it is not clear whether the concentrations of VacA used in our study correspond to the relative concentration on the surface of *H. pylori*-infected host cells. Although VacA concentrations that affect its biological functions in host cells, and physiological concentration during *H. pylori* infection, should be examined in further studies, we propose that VacA does not have a prolonged effect on induction of Src phosphorylation in AZ-521 cells (fig. 1A in Nakano et al., 2016). We observed Src phosphorylation induced by VacA after 1 h incubation with purified VacA, and incubation of AZ-521 cells with VacA for 2 h resulted in diminished signal of Src phosphorylation under some experimental conditions, indicating that Src phosphorylation induced by VacA in AZ-521 cells might be observed at certain points in time. Detailed molecular mechanisms of Src phosphorylation induced by VacA should be evaluated in further studies.

We also think that AGS cells represent a good model gastric epithelial cell line to study the biological functions of CagA in *H. pylori*-infected host cells. Although there appears to be a problem regarding CagA phosphorylation, we used AZ-521 cells because...
this cell line is highly sensitive to VacA-induced actions and expresses several VacA receptors including RPTPα (Isomoto et al., 2010; Radin et al., 2011). But, as Tegtmeyer and Backert mentioned, monolayers of AZ-521 cells and AGS cells might not reflect the precise physiological actions of stomach epithelia. It is difficult to reconstruct detailed environments mimicking host cell surfaces during H. pylori infection using these cell lines. Primary cell cultures and polarized cells with 3D structure, such as organoids generated from the biopsy of human gastric epithelium or animal experimental models, might be applicable for studies regarding the biological functions of virulence factors such as CagA and VacA in H. pylori-infected host cells.

Competing interests
The authors declare no competing or financial interests.

References


Fig. 1. Detection of total CagA and pCagA proteins in AGS and AZ-521 cells. (A) After infection of AGS and AZ-521 cells with H. pylori strain ATCC43504 (WT) at a multiplicity of infection of 100, cell lysates were prepared and loaded on acrylamide gel. Following transfer of proteins to the membrane, signals were detected by standard western blotting. (B,C) After infection of AGS and AZ-521 cells with a WT strain and preparation of cell lysates, immunoprecipitations using anti-CagA antibody were performed to concentrate translocated CagA proteins in host cells as previously described (Nakano et al., 2016). Then, pCagA was detected on the same membrane (B) or on separate membranes (C). To detect the signal in both cell lines, we used 200 µg (AGS) and 500 µg (AZ-521) of whole-cell lysates for immunoprecipitation (C). Signals were detected using anti-phosphotyrosine (pY) and anti-CagA antibodies; α-tubulin served as a loading control. Arrowheads represent the signal of phosphorylated CagA. IB, immunoblotting; IP, immunoprecipitation; PBS, phosphate buffered saline.