

A Novel Inhibitory Domain of *Helicobacter pylori* Protein CagA Reduces CagA Effects on Host Cell Biology^{*[5]}

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Christiane Pelz^{†1}, Sylvia Steininger^{†1}, Claudia Weiss[‡], Fabian Coscia[‡], and Roger Vogelmann^{†§2}

From the [†]Second Department of Internal Medicine, Klinikum rechts der Isar, Technical University Munich, 81675 Munich, Germany and the [‡]Department of Medicine II, University Hospital Mannheim, University of Heidelberg, 68167 Mannheim, Germany

The *Helicobacter pylori* protein CagA (cytotoxin-associated gene A) is associated with an increased risk for gastric cancer formation. After attachment to epithelial cells, the bacteria inject CagA via a type IV secretion apparatus into host cells, where it exerts its biological activity. Host cell responses to intracellular CagA have been linked exclusively to signaling motifs in the C terminus of the CagA protein. Little is known about the functional role of the remaining CagA protein. Using transgenic expression of CagA mutants in epithelial cells, we were able to identify a novel CagA inhibitory domain at the N terminus consisting of the first 200 amino acids. This domain localizes to cell-cell contacts and increases the rate and strength of cell-cell adhesion in epithelial cells. Thus, it compensates for the loss of cell-cell adhesion induced by the C terminus of the CagA protein. Consistent with its stabilizing role on cell-cell adhesion, the CagA N terminus domain reduces the CagA-induced β -catenin transcriptional activity in the nucleus. Furthermore, it inhibits apical surface constriction and cell elongations, host cell phenotypes induced by the C terminus in polarized epithelia. Therefore, our study suggests that CagA contains an intrinsic inhibitory domain that reduces host cell responses to CagA, which have been associated with the formation of cancer.

Helicobacter pylori infection is a well established risk factor for gastric cancer. Epidemiological data suggest that 60–90% of all gastric cancer is attributed to *H. pylori* infection (1, 2). The relative risk for gastric cancer is higher when patients are infected with CagA (cytotoxin-associated gene A)-positive *H. pylori* strains compared with CagA-negative strains (3, 4). Studies in animal models support the epidemiological evidence that CagA is an important virulence factor for gastric cancer. In mongolian gerbils chronic infection with CagA⁺ *H. pylori*, but not a *H. pylori* mutant strain lacking CagA, causes early immunological responses, which eventually leads to precancerous gastric changes (5). Data from transgenic expression of CagA in a mouse model suggest that CagA causes the formation of gas-

tric neoplasms even independent of chronic *H. pylori* infection (6, 7).

CagA is part of the cag pathogenicity island, a set of genomic DNA that also encodes for a type IV secretion system. After attachment of *H. pylori* to epithelial cells, CagA is injected via the type IV secretion system into host cells and consecutively phosphorylated by host Src kinases and c-Abl at tyrosine residues of EPIYA motifs in the C terminus of the protein (8–13). As a result, epithelial gastric carcinoma cells elicit growth factor-like responses such as cell scattering, elongation, and migration (14–18). CagA also has phosphorylation-independent effects on host cell signaling pathways (19–22). CM/CRPIA motifs in the C terminus of CagA contribute to cell scattering and mediate NF- κ B and TCF/ β -catenin³ transcriptional activity (23).

CagA-induced host signaling has been attributed exclusively to signaling motifs located in the C terminus of CagA (24). Little is known about the role of the remaining part, the N terminus of CagA, which accounts for two-thirds of the CagA protein. Bagnoli *et al.* (25) demonstrated that the N terminus of CagA directs the protein to the plasma membrane of epithelial cells independent of the C terminus. However, data regarding the mechanism of CagA interaction with the epithelial membrane appear to be inconsistent. Higashi *et al.* (16, 26) described that the EPIYA motifs in the C terminus are required for membrane attachment, thereby initiating EPIYA-induced host signaling.

Therefore, we asked the question how the N terminus of CagA affects host cell physiology, both dependent and independent of signaling motifs in the CagA C terminus. In this manuscript we present data showing that CagA consists of two independent domains at the N and C termini of the protein, respectively, with which the protein tethers to structures at the plasma membrane of host cells. The first 200 AA of the N terminus of CagA act as an inhibitory domain of host cell responses evoked by the CagA C terminus: (i) it increases the rate and strength of newly formed cell-cell contacts, (ii) it decreases cell elongation and constriction of the apical membrane induced by the C terminus, and (iii) it reduces TCF/ β -catenin transcriptional activity mediated by the C terminus of CagA.

EXPERIMENTAL PROCEDURES

Cell Lines—Mardin-Darby canine kidney (MDCK) II cells were cultured in DMEM (Invitrogen) containing 10% fetal

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¹ Both authors contributed equally to this work.

² To whom correspondence should be addressed: Ismaninger Str. 22, 81675 Munich, Germany. Tel.: 49-89-4140-6716; Fax: 49-89-4140-2258; E-mail: roger.vogelmann@lrz.tum.de.

³ The abbreviations used are: TCF, T-cell factor; MDCK, Mardin-Darby canine kidney; ms, mouse; mc, monoclonal; AA, amino acid(s); RFP, red fluorescent protein.

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bovine serum as described before (27). For MDCK II cells to polarize, 5×10^5 cells were plated on Transwell filters (12-mm well, 0.4- μ m pore size; Corning) and incubated for 2 days before transfection experiments. The gastric cell line NCI-N87 was obtained from American Type Culture Collection and maintained in recommended culture conditions. Lipofectamine LTX (Invitrogen) was used for transfection of plasmid cDNA according to the manufacturer's manual. Inducible expression of CagA and CagA mutants in MDCK II cell lines was generated via the Tet-On[®] advanced inducible gene expression system (Clontech). For induction of protein expression, 3 μ g/ml doxycycline was added to cell culture medium 24 h prior to experiments.

Plasmids—For CagA cloning, we modified the pTRE-tight vector from Clontech as follows. The multiple cloning site was replaced by enhanced GFP from Clontech vector pEGFP at the N terminus and the SBP/CBP tag from vector pCTAP (Stratagene) at the C terminus. CagA constructs were generated by PCR from previously published pEGFP-CagA WT plasmid (*H. pylori* strain G27 (25)). CagA mutants 1–200, 1–150, 27–225, and 200–800 were cloned in-frame between GFP and SBP/CBP via Sall/NotI. CagA mutants 200–1216, 400–1216, and Δ 200–800 were cloned by replacing the SBP/CBP tag with a CagA 800–1216 PCR product via NotI/XbaI. Respective CagA PCR products 200–800, 400–800, 1–200, and 27–800 were cloned in-frame between GFP and CagA 800–1216 via Sall/NotI. For co-expression experiments, CagA 871–1216 was cloned by replacing the SBP/CBP tag with a respective PCR product via NotI/XbaI. For RFP tagging of CagA 871–1216 and CagA 200–1216, enhanced GFP was replaced by monomeric RFP (28). CagA WT was inserted in-frame via Eco47III/NotI into a pTRE-Myc vector (Clontech) with the modified multiple cloning site (25). In a second step, GFP-CagA WT was cloned via BamHI/NotI into the above-mentioned modified pTRE-tight vector and therefore includes in addition an N-terminal Myc tag. For CagA EPISA C, a chemically synthesized cDNA fragment encoding for AA 943–1055 with both EPIYA C motifs mutated to EPISA C was inserted into CagA Δ Par1 via AscI/XbaI. CagA Δ Par1 was cloned by replacing AA 943–1027 with an AscI restriction site in CagA WT. Because of technical requirements, synthesis of the EPISA C fragment required a change in codon usage (from *Canis familiaris*) optimized by GENEius software (Eurofins) (supplemental Fig. S1).

Immunofluorescence—For confocal immunofluorescence microscopy, the cells were fixed with 2% paraformaldehyde in 100 mM sodium phosphate buffer, pH 7.4, and were permeabilized in PBS + 3% bovine serum albumin + 1% saponin + 0.1% Triton X-100 for 10 min at room temperature. The cells were stained with antibodies against ZO-1 (ms, mc, 1:200), Alexa Fluor 594- or 647-coupled phalloidin (Invitrogen) and anti- β -catenin (ms, mc, 1:200; BD Bioscience). Secondary antibodies (anti-mouse Alexa Fluor 546 and 647) were purchased from Invitrogen. The samples were imaged with a confocal microscope (Leica SP5), and z-stacks were projected onto three-dimensional reconstructions by using Volocity 4.1 software (PerkinElmer Life Sciences). The figures were assembled with Photoshop CS (Adobe Systems).

Membrane Pelleting Assay and Immunoblotting—MDCK II cells stably expressing CagA mutants (1×10^8 cells in two 150-mm dishes) were washed three times with PBS before transfer into detergent free homogenization buffer (20 mM Hepes-KOH, pH 7.2, 90 mM potassium acetate, 2 mM magnesium acetate, 25 mM sucrose, proteinase inhibitors, and phosphatase inhibitor mixture 2 from Sigma-Aldrich). The cells were mechanically broken via Branson sonifier 250 at 4 °C (duty cycle, 50%; output control at Microtip limit, level 1 for 15 s; pause, 1 min; level 2 for 10 s). After centrifugation at $100,000 \times g$ for 45 min at 4 °C in a Beckman Coulter Type 100 Ti rotor, supernatant was removed, and pellet was resuspended in equal volumes of homogenization buffer. Respective protein samples were boiled in SDS sample buffer containing dithiothreitol (final concentration, 50 mM) for 10 min and separated in SDS-polyacrylamide gels as described previously (29). After transfer to Immobilon-FL 0.45-m polyvinylidene fluoride membranes (Millipore), the proteins were blocked with LI-COR blocking buffer (Li-Cor, Germany) in PBS (1:1) for 1 h at room temperature. Primary antibodies and fluorescence-labeled secondary antibodies (1:30,000) were diluted in T-PBS (PBS + 0.1% Tween 20) and incubated for 1 h at room temperature. The membranes were scanned with Odyssey infrared imaging system (Li-Cor) at 680- and 800-nm wavelengths, and the amount of protein per lane was determined with Odyssey software 1.2. Primary antibody against CagA (rabbit, polyclonal, 1:3000) was made against recombinant GST-CagA1–877 fusion protein expressed in *Escherichia coli* (30). Anti-E-cadherin (ms, mc, 1:3000) was purchased from BD Bioscience, anti-actin (ms, mc, 1:1000) was from Sigma-Aldrich, and anti-Mek-1 (clone H-8, ms, mc, 1:1000) was from Santa Cruz. Alexa-Fluor 680 goat anti-mouse was purchased from Invitrogen, and anti-rabbit IRDye 800 was from Rockland Immunochemicals.

Apical Constriction and Cell Elongation Assay—Polarized MDCK II cells were transiently transfected with CagA and CagA mutant plasmids. To measure apical surface area, we stained epithelial cells with antibodies against ZO-1 and identified transfected cells via GFP fluorescence signal. Confocal optical sections from random fields were collapsed into single projections. Apical surface areas of individual cells were determined as the area confined by ZO-1 and measured using ImageJ software (National Institutes of Health). For the cell elongation assay, CagA or CagA mutant transfected cells were evaluated for cellular protrusion formation. Protrusions extending the diameter of the main cell body were counted as positive for cell elongation. CagA-expressing cells were evaluated independent of their expression level, and the numbers of cells were counted as indicated in the figure legends.

Hanging Drop Adhesion Assay—The assay was performed as described before (31). In brief, MDCK II cell lines stably expressing CagA or CagA mutants were grown at low density, and expression was induced 24 h before the experiment. The cells were trypsinized, centrifuged, and resuspended as single-cell suspensions at 2.5×10^5 cells/ml. Twenty-microliter drops of cell suspension were pipetted onto inside surface of six-well cell culture dish lids, and the wells were filled with 2 ml of medium each to prevent evaporation. At 4 h, the lid was inverted, and the drops were spread onto a glass slide. For trit-

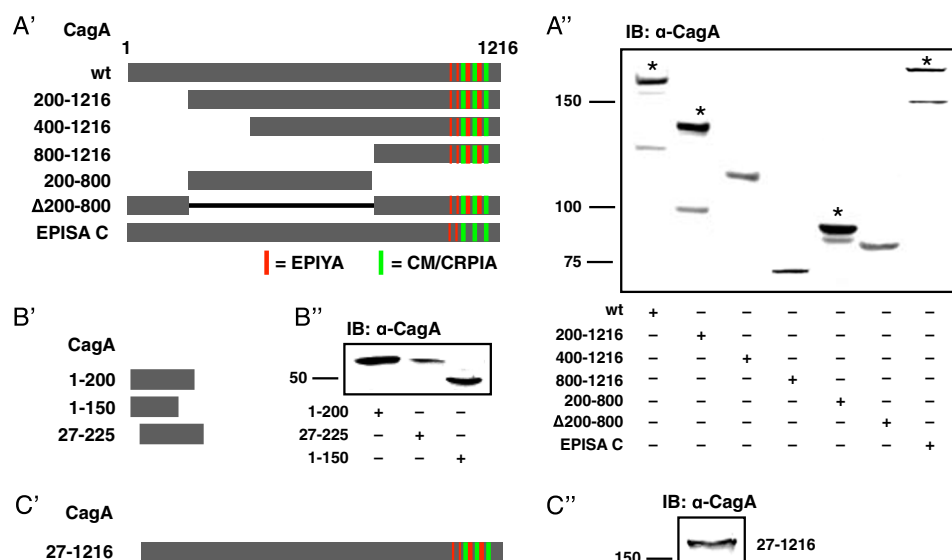


FIGURE 1. **CagA and CagA mutants.** A', B', and C', maps of CagA WT and mutants. A'', B'', and C'', immunoblots (IB) of CagA WT and mutants. *, specific bands A'. The size difference between CagA 200–800 and Δ200–800 is due to an additional SBP/CBP tag in CagA 200–800.

uration, the drops were first pipetted 10 times through a 20- μ l pipette. At each time point, three drops were photographed, and the numbers and sizes of clusters were determined.

TCF/ β -Catenin Transcriptional Activity—NCI-N87 cells were cultured in 24-well plates and grown to 60% confluence. The cells were co-transfected with 100 ng of Topflash, 100 ng of Fopflash (kindly provided by W. J. Nelson, Stanford University), 100 ng of pTet-Advanced (Clontech), and 300 ng of CagA WT or CagA 200–1216 cDNA, respectively. CagA expression was activated using 3 μ g/ml doxycycline. After 24 h, the cells were harvested in 100 μ l of reporter lysis buffer (Promega), and luciferase activity was determined in a dual channel luminometer according to manufacturer's protocol for a dual luciferase reporter assay system (Promega). The results were normalized for transfection efficiency by co-transfection of 6.25 ng of *Renilla* luciferase plasmid (kindly provided by W. J. Nelson, Stanford University).

Statistics—The mean values and S.E. were calculated from at least three independent experiments. For statistical analysis, one-way analysis of variance, Student's *t* test, Tukey's multiple comparison test, Cochran-Mantel-Haenszel test, and Wilcoxon rank-sum test were used as indicated in figure legends (R project for statistical computing, R version 2.8.1 for Mac OS X, GNU Project, and GraphPad Prism, Graph Pad Software).

RESULTS

Because CagA targeting to the membrane is important for host signaling (16, 25, 26), we re-evaluated the capacity of various truncated CagA mutants (Fig. 1) to target the protein to the epithelial plasma membrane. The CagA sequence was derived from the *H. pylori* strain G27, and CagA mutants were expressed in MDCK II cells as a model for polarized epithelial cells (25, 32, 33). All of the CagA constructs were tagged with GFP at the N terminus of CagA and transiently or stably expressed in a doxycycline-inducible gene expression system. By the addition of doxycycline to the growth medium, CagA expression is turned on within hours.

CagA C Terminus Directs CagA to the Host Cell Membrane Compartment—It has been reported that CagA derived from the *H. pylori* NCTC11637 strain interacts with the plasma membrane via the EPIYA motifs of CagA in undifferentiated epithelial gastric cancer cells. A *cagA* deletion mutant lacking the EPIYA motifs in the C terminus did not co-localize with the membrane compartment in AGS cells (26). Therefore, we examined whether the EPIYA motif containing the C terminus is sufficient for this interaction. In our study, a CagA mutant containing the EPIYA motifs (CagA 800–1216) localized in the cytoplasm when transiently transfected in polarized MDCK II cells (Fig. 2A). This confirms the finding in a previous study using a CagA 871–1216 mutant derived from the same *H. pylori* strain G27 (25). Because a cytoplasmic immunofluorescence signal could mask co-localization with the membrane compartment, we analyzed the distribution of CagA 800–1216 between membrane and cytoplasm in a biochemical membrane pelleting assay using MDCK II cells stably expressing CagA and CagA mutants under the control of a doxycycline-inducible gene expression system.

In this assay, the cells were broken mechanically in a buffer without detergents to preserve lipid membrane particles. This was followed by high speed ultracentrifugation, which pellets particles and separates them from the soluble content of the cytoplasm. A marker for plasma membrane, E-cadherin, is localized in the pellet fraction, whereas a marker for cytoplasmic proteins, Mek-1, is in the supernatant (Fig. 2B). Approximately two-thirds of the CagA 800–1216 mutant protein were detected in the membrane fraction (Fig. 2B), indicating an indirect or direct interaction of this part with the membrane compartment. These data suggest that the EPIYA-containing C terminus interacts with membrane fractions but is not sufficient for a complete membrane association in epithelial cells.

To determine which other parts of CagA are necessary for a complete co-localization with the membrane compartment, we tested CagA mutants varying in length at the C terminus. A

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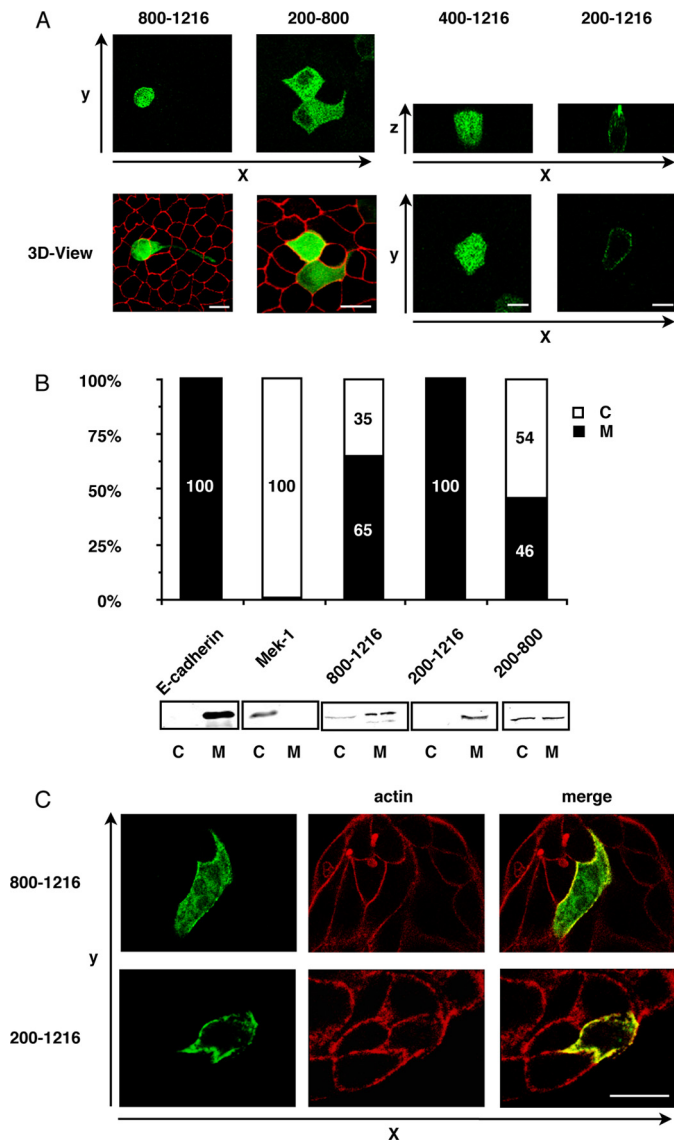


FIGURE 2. C-terminal CagA membrane-targeting domain. A, cellular distribution of CagA mutants in MDCK II cells. Shown are three-dimensional reconstructions of confocal z-stacks (3D-View) and representative x-y or x-z planes of corresponding confocal z-stacks. Green, GFP-CagA mutants; red, ZO-1. B, membrane pelleting assay. The signal intensity for each protein band was determined as integrated intensity (counts/mm²) and expressed as percentages of the sum of integrated intensities in membrane (M) and cytoplasm (C). C, cellular distribution of CagA mutants in NCI-N87 cells. Shown are representative x-y planes of confocal z-stacks. Green, GFP-CagA mutants; red, actin; bar, 10 μ m.

CagA mutant consisting of AA 400–1216 (CagA 400–1216) was similar to CagA 800–1216 in its subcellular localization (Fig. 2A). In contrast, an extended CagA mutant AA 200–1216 (CagA 200–1216) localized exclusively to the membrane compartment as assessed by immunofluorescence with a strong localization to the apical membrane compartment in polarized epithelial cells (Fig. 2A). In the membrane pelleting assay, CagA 200–1216 localized completely with the membrane fraction, confirming the immunofluorescence result (Fig. 2B).

The ability of CagA 200–1216 to localize to the membrane is not dependent on the CagA segment 200–400 alone, because a CagA 200–800 fragment is distributed similarly to CagA 800–1216 throughout the cytoplasm when assessed by immunofluorescence staining (Fig. 2A). The high speed centrifugation step revealed that ~46% of the mutant protein associated with the membrane compartment (Fig. 2B).

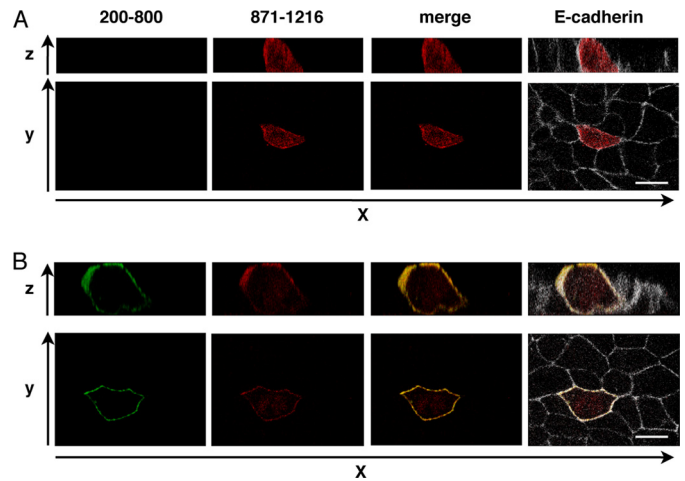


FIGURE 3. CagA 200–800 and 871–1216 interact in trans. Shown are MDCK II cells transiently expressing mRFP-CagA 871–1216 (red) alone (A) or together with GFP-CagA 200–800 (green) (B). Shown are representative x-y and x-z planes of confocal z-stacks, E-cadherin (white). Bar, 10 μ m.

orecence staining (Fig. 2A). The high speed centrifugation step revealed that ~46% of the mutant protein associated with the membrane compartment (Fig. 2B).

To show that the cellular localization of the CagA C-terminal mutant proteins is independent of cell lines, we expressed the CagA fragments transiently in the gastric epithelial cell line NCI-N87. Both analyzed constructs, CagA 800–1216 and CagA 200–1216, behaved similarly in regard to their cellular distribution in these cells as shown for MDCK II cells (Fig. 2C).

A previous report demonstrated that a N terminus fragment of CagA (AA 1–877) interacts with the C terminus fragment (AA 871–1216) when transiently expressed in *trans* in epithelial cells. Thus, the C-terminal part (AA 871–1216) is directed from the cytoplasm to the membrane compartment (25). Therefore we asked whether the CagA fragments AA 200–800 and AA 871–1216 are able to interact with each other. The localization of RFP-CagA 871–1216 in transiently transfected polarized MDCK II cells is similar to GFP-CagA 800–1216 (compare Figs. 3A and 2A). When ectopically expressed in the same cell, the mutant proteins GFP-CagA 200–800 and RFP-CagA 871–1216 co-localized with each other primarily at the membrane compartment (Fig. 3B). These data suggest that CagA 200–800 and the EPIYA motifs containing C terminus of CagA (871–1216) interact with each other, forming a domain that targets the CagA protein directly or indirectly to the membrane compartment.

CagA N Terminus Directs CagA to the Host Cell Membrane Compartment—It has been published that the N terminus of CagA (CagA 1–877) interacts with the plasma membrane independent of the CagA C terminus (25). Because CagA 200–800 located only partially to the membrane compartment (Fig. 2, A and B), we analyzed the first 200 AA of the N terminus in regard to its capacity to co-localize with the membrane compartment.

Transient expression of a CagA 1–200 fragment in polarized MDCK II cells reveals that this fragment is sufficient to co-localize with the membrane compartment (Fig. 4A). The cellular distribution was again confirmed using NCI-N87 cells (Fig. 4A).

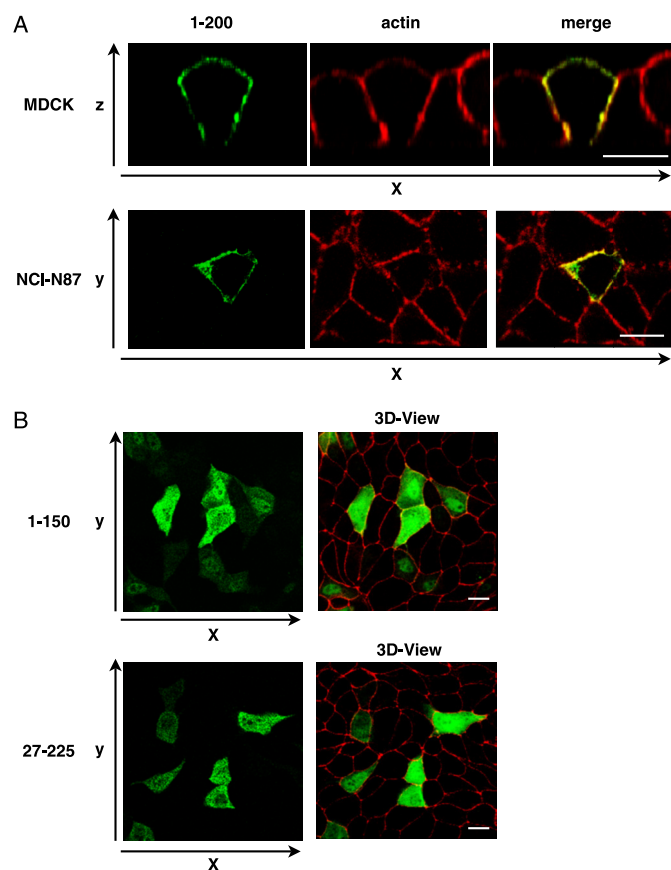


FIGURE 4. N terminus CagA membrane-targeting domain. *A*, confocal microscopy images (representative *x-y* or *x-z* planes) of transient expression of GFP-CagA 1–200 (green) in polarized MDCK II and NCI-N87 cells, actin (red). Bar, 10 μ m. *B*, GFP-CagA 1–150 and 27–225 (green) in polarized epithelial cells (MDCK II). Shown are three-dimensional reconstructions of confocal *z*-stacks (3D-View) and representative *x-y* planes, ZO-1 (red). Bar, 10 μ m.

The ability of the N terminus of CagA to co-localize with the plasma membrane in epithelial cells is confined to the first 200 AA. CagA mutants shorter than 200 AA (CagA 1–150) or a mutant lacking the first 26 AA (CagA 27–225) were distributed throughout the cytoplasm (Fig. 4*B*).

When expressed in nonpolarized subconfluent MDCK II cells, CagA 1–200 was enriched at sites of cell-cell contacts co-localizing with β -catenin, a protein important for the formation of cell-cell adhesion (Fig. 5*A*). CagA 1–200 was excluded from membrane sites not engaged in cell-cell contacts (see *arrowhead* in Fig. 5*B*). Interestingly, in nonpolarized subconfluent cells, the C terminus fragment CagA 200–1216 distributes along the entire membrane compartment including membrane sites not engaged in cell-cell contacts (see *arrowhead* in Fig. 5*C*). This diverse distribution pattern of the N-terminal and C-terminal domain at the membrane suggests that they may interact with different membrane substructures.

Impact of Membrane-targeting Domains on CagA-induced Cell Elongation—After identifying two distinct membrane-targeting domains, we asked whether they would interfere with each other's role in host cell responses. Once CagA is intracellular in host cells, it causes cell elongation (14, 15, 18). Bagnoli *et al.* (25) showed that in polarized epithelial cells CagA-express-

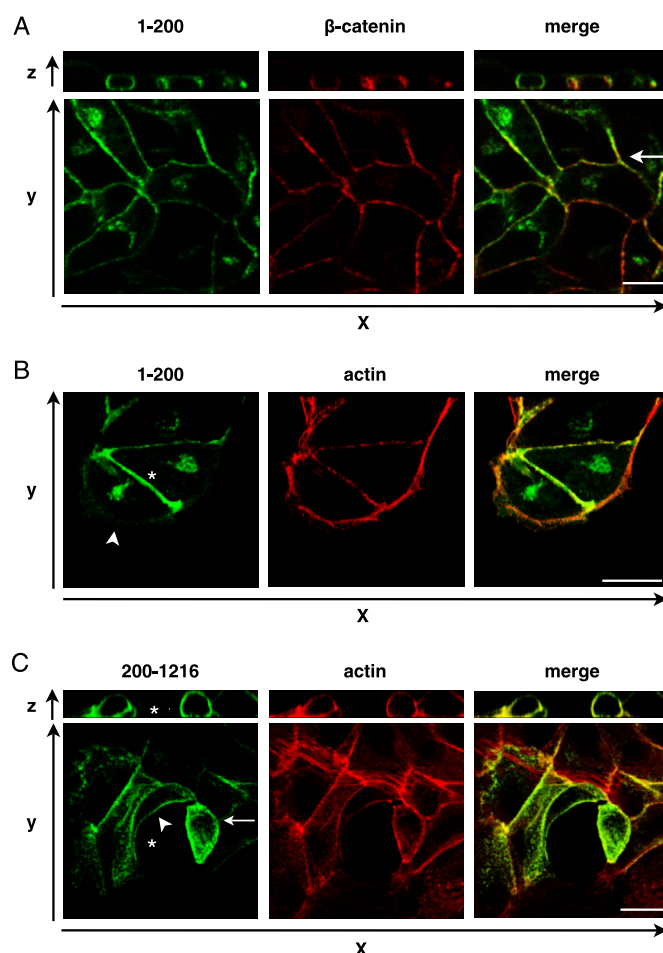


FIGURE 5. CagA membrane-targeting domains in nonpolarized cells. *A*, CagA 1–200 (green) in nonpolarized MDCK II cells; co-localization with β -catenin (red) at lateral membrane. White arrow indicates plan of *z*-section of corresponding *x-y* plane. *B*, *, enriched at cell-cell contacts; arrowhead, excluded from free edge; red, actin. *C*, green, CagA 200–1216 in nonpolarized MDCK II cells; *, surface area free of cells; arrowhead, CagA 200–1216 enriched at free edge of cell; red, actin; white arrow, *z*-section of corresponding *x-y* plane; bar, 10 μ m.

ing host cells exhibit an elongated morphology with a constriction of the apical surface area.

To analyze the effects of both membrane-targeting domains on cell elongation, we counted the number of CagA expressing cells with cellular protrusions extending the diameter of the cell body in transiently transfected polarized MDCK II cells (Fig. 6*A*). Host cells expressing CagA WT (1–1216) developed cellular protrusions in $24.7 \pm 4\%$ of transfected cells (Fig. 6*B*). However, the formation of cellular protrusions was significantly increased to $48.2 \pm 1.3\%$ in CagA 200–1216 expressing cells. The number of CagA 800–1216-induced cellular protrusions was similar to CagA 200–1216 ($46 \pm 3.3\%$). CagA 1–200 had no effect on cell elongation (Fig. 6*B*). The decrease of cell elongation in CagA WT compared with CagA 200–1216 expressing cells suggests that CagA 1–200 exerts an inhibitory effect on cell signaling mediated by the C terminus of CagA.

CagA 1–200 does not exert its inhibitory effect by acting directly on CagA 200–1216. When co-expressed as two separate mutants in the same cell, CagA 1–200 does not inhibit

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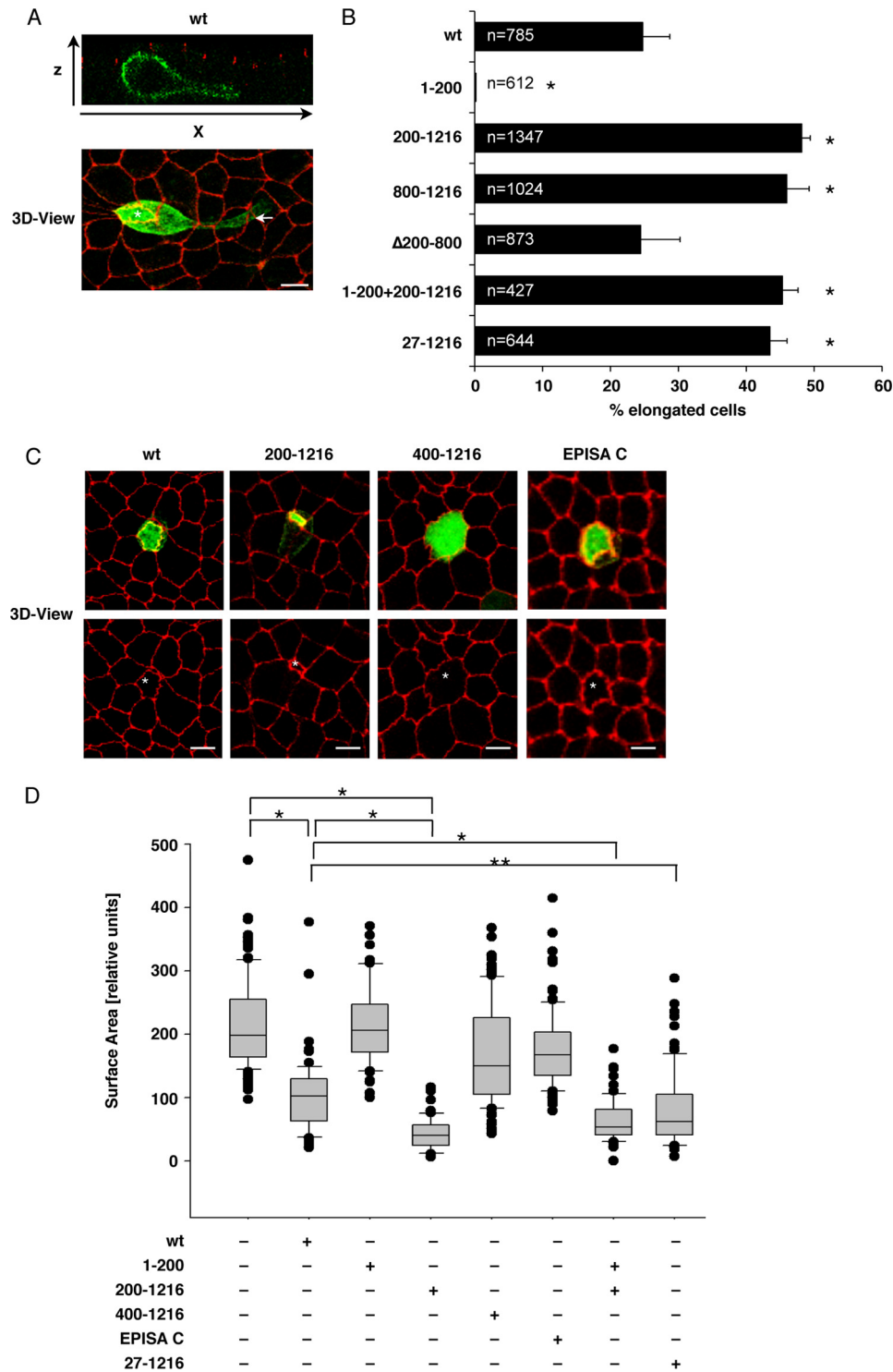


FIGURE 6. CagA 1–200 membrane-targeting domain inhibits cell elongation and apical surface constriction. *A*, CagA WT (green) induces cell elongation (white arrow) and constriction of apical surface area (*) in polarized MDCK II cells. Shown are three-dimensional reconstruction of confocal z-stacks (3D-View) and representative x-z plane, ZO-1 (red). Bar, 10 μ m. *B*, percentage of elongated cells transiently transfected with CagA WT or CagA mutants in polarized epithelia; the data are presented as the means \pm S.E. *, $p < 0.05$, (Student's *t* test). *n* indicates number of cells counted in three to six independent experiments. *C*, apical constriction of CagA mutants. Three-dimensional reconstruction of confocal z-stacks is shown. *, apical surface area of CagA expressing cells; green, GFP-CagA WT, GFP-CagA mutants; red, ZO-1; bar, 10 μ m. *D*, box plot graph of apical surface area: control $n = 138$, WT $n = 65$, 1–200 $n = 61$, 200–1216 $n = 87$, 400–1216 $n = 100$, EPISA C $n = 92$, 1–200 + 200–1216 $n = 85$, 27–1216 $n = 89$ cells. *, $p < 0.0001$; **, $p < 0.05$ (Wilcoxon rank-sum test).

CagA 200–1216-induced cell elongation ($45.3 \pm 2.3\%$) (Fig. 6*B*). However, covalent binding to the signaling motifs in the C terminus is required for the inhibitory effect. CagA 1–200, when fused to CagA 800–1216 (CagA Δ 200–800), tethers

the CagA C terminus to the cell membrane (compare [supplemental Fig. S2A](#) and Fig. 2*A*). Epithelial cells expressing CagA Δ 200–800 elongated significantly less compared with CagA 800–1216 but similar to CagA WT ($24.4 \pm 5.8\%$)

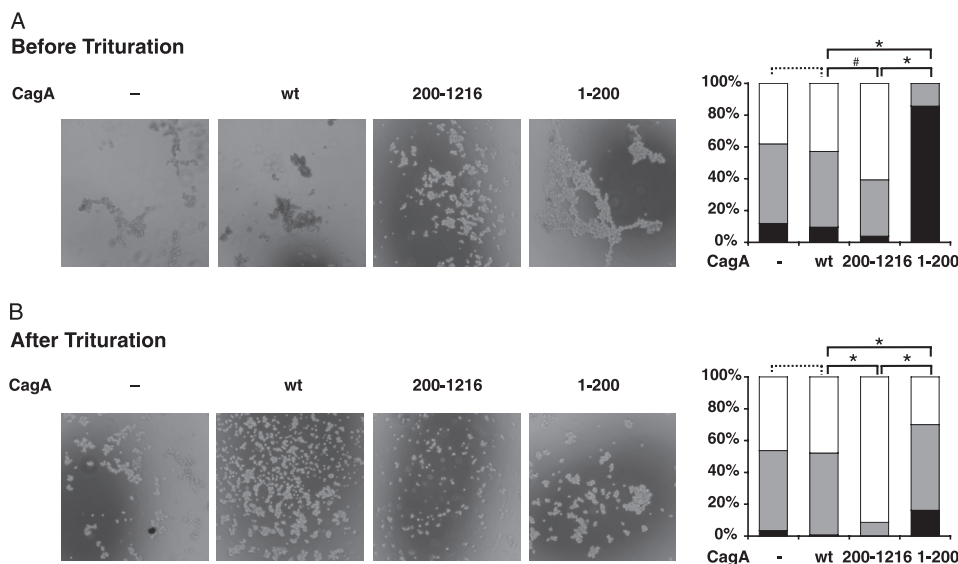


FIGURE 7. Opposite effects of CagA membrane-targeting domains on cell-cell adhesion. Quantitative, functional adhesion assay of MDCK II cells stably expressing CagA WT, 200–1216 and 1–200 mutants. Control represents pooled data from noninduced MDCK II cells of all three clones. Graphs show percentages of cells in clusters of 0–10 cells (*white*), 11–50 cells (*gray*), and >50 cells (*black*) after 4 h before (A) and after (B) trituration. The data are presented as the averages of three independent experiments; ..., not significant; *, $p < 0.0001$; #, $p < 0.05$ (Cochran-Mantel-Haenszel test). The photographs are representative fields.

(Fig. 6B). These data suggest that the inhibitory effect of CagA 1–200 is due to membrane targeting of the C-terminal signaling motifs to an alternative membrane substructure.

Because the deletion of larger parts of a protein could cause conformational changes, we analyzed the much shorter deletion mutants of CagA (CagA 27–1216). The deletion of the first 26 AA of the CagA 1–200 fragment led to a loss of the membrane-targeting domain at the N terminus as shown in Fig. 4B. The mutant still localized to the host cell membrane, not because of the N-terminal but likely because of the C-terminal membrane-targeting domain (supplemental Fig. S2B). CagA 27–1216 induced cellular protrusions in $43.5 \pm 2.5\%$ of expressing host cells similar to CagA 200–1216 (Fig. 6B). This further supports our hypothesis that CagA 1–200 exerts its inhibitory effect because of targeting of C terminus signaling motifs to an alternative membrane substructure.

CagA expression in polarized epithelial cells causes a constriction of the apical surface area (Fig. 6C) (25). We analyzed the surface area of CagA and CagA mutant expressing polarized cells. Expression of CagA 200–1216 showed a strong constriction of the apical surface area compared with nonexpressing control cells (median surface area, 40 *versus* 199 relative units), whereas CagA 1–200 had no effect on apical constriction (median surface area, 206 relative units; Fig. 6D). However, apical constriction exerted by CagA WT was significantly reduced compared with CagA 200–1216 (median surface area, 102 *versus* 40 relative units), which suggests that CagA 1–200 interfered with CagA 200–1216-mediated constriction of the apical surface area in polarized epithelia. The subcellular localization of CagA 200–1216 to the apical membrane and phosphorylation at the EPIYA C motifs are important for apical constriction. The CagA 400–1216 mutant, which localizes less to the membrane compartment (Fig. 2A), and CagA EPISA C, a mutant that cannot be phosphorylated at the EPIYA C motif,

did not constrict the apical surface area, respectively (median surface area, 165 and 176 relative units, respectively; Fig. 6D).

Again, covalent binding of CagA 1–200 to signaling motifs in the C terminus was required for the inhibitory effect. Co-expression of CagA 1–200 and CagA 200–1216 as two separate mutants in the same cell did not inhibit the phenotype of CagA 200–1216-induced constriction of the apical surface area (median surface area, 63 *versus* 40 relative units).

The CagA mutant 27–1216, a shorter deletion mutant to minimize the impact of conformational changes, also did not reduce CagA 200–1216-induced apical surface constriction (median surface area, 80 relative units). Our data suggest that the inhibitory effect of the N-terminal domain CagA 1–200 is due to its tethering ability to a different membrane compartment.

Impact of Membrane-targeting Domains on Cell-Cell Adhesion—CagA-induced loss of cell-cell adhesion is a distinct host cell response (15, 18, 25, 34, 35). Therefore, we analyzed whether the membrane-targeting domain CagA 1–200 affects cell-cell adhesion using a hanging drop adhesion assay, which determines the size of cell clusters formed over time from single cells in suspension. The application of shearing forces through trituration reveals the strength of newly formed adhesion complexes (31).

For this cell-cell adhesion assay, we used MDCK II cells stably expressing CagA and CagA mutants under the control of a doxycycline-inducible gene expression system. These mutants were expressed at comparable levels in ~80% of cells (supplemental Fig. S3). The experiments were performed in triplicate with 200–400 cells examined 4 h after forming a single cell suspension using noninduced cells as control. In control cells, 62% of clusters consisted of more than 10 cells/cluster (sum of *gray* and *black areas* in Fig. 7A). Interestingly, cluster formation with more than 10 cells/cluster in CagA WT-expressing cells

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was not significantly different from control cells (57%) but significantly decreased to 39% in CagA 200–1216 mutant cells. However, expression of the CagA 1–200 membrane-binding domain increased the rate of cell cluster formation to 100% of clusters with 10 or more cells (Fig. 7A).

Applying shearing forces to the clusters revealed that the CagA 1–200 domain also increased the strength of cell-cell adhesion in formed cell clusters (Fig. 7B). After trituration of cell clusters formed after 4 h, the number of large cell clusters (10–50 and >50 cells/cluster group) was similar between CagA WT and control cells and significantly increased in CagA 1–200 expressing cells (52% versus 54% versus 70%, respectively). Epithelial cells expressing the CagA 200–1216 mutant formed dramatically weaker cell-cell adhesions (9%). These data show that the CagA C terminus membrane-targeting domain 200–1216 mediates the loss of cell-cell adhesion and that CagA 1–200 counteracts this effect by increasing the formation and strength of newly formed cell-cell contacts.

Impact of Membrane-targeting Domains on Transcriptional Activity of β -Catenin—Loss of cell-cell adhesion has been linked to the ability of CagA to increase transcriptional activity of β -catenin (17, 34, 36). Cell-cell adhesion is mediated by adherens junctions, which consist of the transmembrane protein E-cadherin binding directly to β -catenin. This interaction is stabilized when cell-cell contacts are established (37). A different role of β -catenin is its function as a transcriptional regulator of gene expression in the nucleus by binding to TCF/lymphoid enhancer factor transcription factors (38). CagA has been described to disrupt the E-cadherin/ β -catenin complex leading to a weakening of cell-cell adhesion and to an increase of transcriptional activity of β -catenin (17, 34, 36). CagA induced an increase in TCF/ β -catenin transcriptional activity, which is mediated by the CM/CRPIA motif in the C terminus of CagA (23), has been shown in cell lines with a constitutive TCF/ β -catenin transcriptional activity (34, 36). In our model for polarized epithelial cells, TCF/ β -catenin-mediated transcription is not constitutively activated (39), and CagA WT did not alter TCF/ β -catenin transcriptional activity (TOP) compared with the control with a mutated TCF-binding site (FOP) (supplemental Fig. S4). Therefore, we tested the impact of CagA WT and the CagA 200–1216 membrane-targeting domain on β -catenin transcriptional activity in the gastric epithelial cell line NCI-N87, which forms adherens junctions and has constitutive TCF/ β -catenin transcriptional activity (40). The CagA 1–200 and CagA 200–1216 mutants behaved similarly in regard to membrane targeting in these cells as shown for MDCK II cells (Figs. 2 and 4). The baseline transcriptional activity of β -catenin is increased in NCI-N87 cells (Fig. 8). The CM/CRPIA motif containing CagA mutant 200–1216 increased TOP luciferase activity as a read-out for β -catenin transcriptional activity by 2.65-fold to control cells ($p < 0.0001$). Consistent with its stimulating effect on cell-cell adhesion, the CagA 1–200 membrane-binding domain bound to CagA 200–1216 attenuated CagA 200–1216-induced β -catenin transcriptional activity by 27% because CagA WT-induced TOP luciferase activity was only 1.93-fold compared with control ($p < 0.0001$).

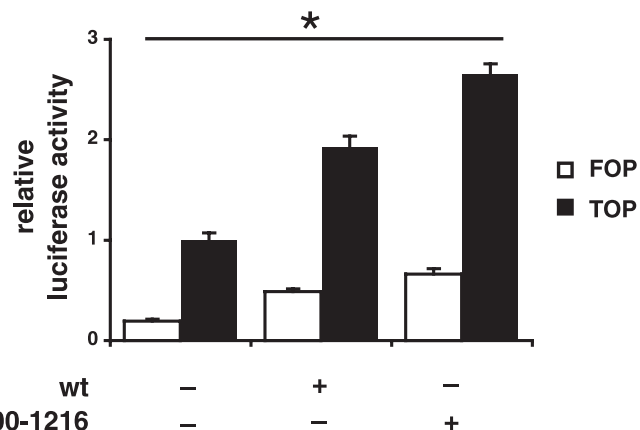


FIGURE 8. CagA 1–200 bound to CagA 200–1216 decreases TCF/ β -catenin transcriptional activity of the C terminus. Shown is the TCF/ β -catenin transcriptional activity of NCI-N87 cells transiently transfected with CagA WT, CagA 200–1216, or empty vector. The data represent the means \pm S.E. calculated from three independent experiments as x-fold induction compared with activity of reporter vector in the absence of CagA. *, $p < 0.0001$ (one-way analysis of variance; Tukey's multiple comparison test); black, TOPflash; white, FOPflash.

DISCUSSION

It has been demonstrated before that targeting of CagA to the membrane is an essential prerequisite for the effects of CagA on host cells. CagA specifically binds to and activates Src homology 2-containing protein-tyrosine phosphatase-2 at the membrane via EPIYA motifs in the C terminus of CagA, thereby inducing cell elongation in host epithelial cells. Membrane targeting of activated Src homology 2-containing protein-tyrosine phosphatase-2 is necessary and sufficient for the induction of this phenotype (16, 26). In a second study in polarized epithelial cells, membrane targeting of CagA was important for detachment of host cells from neighboring cells during cell migration (25).

Despite the significance of CagA membrane targeting for its effect on host cell signaling, until now only two studies have addressed the question of which domains of CagA are necessary for its tethering to the membrane compartment. The results appeared to be inconsistent at first glance (25, 26): a CagA mutant lacking EPIYA motifs in the C terminus of CagA (CagA Δ 868–1087) no longer co-localized with the membrane compartment. These data showed that EPIYA motifs are required for CagA membrane targeting (26). In contrast, Bagnoli *et al.* (25) showed that a CagA mutant lacking the entire C terminus including EPIYA motifs (CagA Δ 878–1216) co-localized entirely with the plasma membrane, suggesting that membrane targeting of CagA is independent of EPIYA motifs. In addition, the EPIYA motif containing C terminus of CagA (CagA 871–1216) alone is not sufficient to co-localize with the membrane (25). The difference could be due to sequence divergence in CagA (*H. pylori* strain NCTC11637 versus G27), host cell variations of used cell lines (AGS versus MDCK II cells), or the experimental set-up evaluating membrane targeting by immunofluorescence microscopy. Data from our study give further insight into the mechanism of membrane targeting.

Using immunofluorescence analysis, we confirmed the finding by Bagnoli *et al.* (25) that CagA 800–1216 alone localizes to

the cytoplasm. However, using a biochemical membrane pelleting assay, we could show that approximately two-thirds of the protein can still be detected in the membrane fraction, suggesting that CagA 800–1216 interacts to some degree with membranes and the immunofluorescence signal in the cytoplasm masked co-localization with the membrane compartment. For a complete membrane targeting of the C terminus, amino acids 200–1216 are required. Segments 200–800 and 800–1216 interact with each other and seem to form a domain that is responsible for co-localization with the membrane compartment. Neither of them alone has this capacity for complete membrane targeting.

In addition, we were able to show in this study that CagA exhibits a second membrane-targeting domain. Previously published data showed that a CagA mutant consisting of the first 600 AA localizes to the plasma membrane of epithelial cells (25). We were able to narrow down this second membrane-targeting domain to the first 200 AA of the N terminus. Because CagA 27–225 and CagA 1–150 are no longer able to localize to the membrane, CagA 1–200 is required for its targeting ability.

Membrane targeting has an important impact on host cell responses, hence interaction with host cell proteins. Twenty different host cell proteins have been identified so far to interact directly or indirectly with CagA (24). Most of these proteins interact with signaling motifs in the C terminus of CagA like EPIYA and CM/CRPIA motifs (23, 41, 42). Early in the course of infection, CagA is interacting with the host membrane component phosphatidylserine close to the injection site at the membrane likely at the apical membrane of polarized epithelia (33, 35, 43). However, many of the host signaling proteins like Par-1, focal adhesion kinase, E-cadherin, ZO-1, and JAM-1, with which CagA interacts, are confined to basal-lateral membranes in polarized epithelia (32, 44–46). Different hypotheses have been proposed regarding how CagA could interact with proteins of the basal-lateral membrane compartment. *H. pylori* infection may cause localization of basal-lateral membrane proteins to the apical membrane prior to CagA injection (47), or CagA-induced loss of cell polarity causes mislocalization of proteins to the apical compartment, which would otherwise be confined to basal-lateral membranes (33, 41). Our data lead to the suggestion that the second membrane-targeting domain would give a bacterial effector protein the ability to localize to a membrane compartment away from the injection site so that it can recruit and interfere with other host-signaling proteins. In subconfluent cells, the N terminus membrane-targeting domain CagA 1–200 co-localizes mainly with membranes engaged in cell-cell contacts, whereas CagA 200–1216 also co-localizes with membranes that are not engaged. This suggests that the CagA membrane-targeting domains interact with different membrane substructures.

The CagA 1–200 domain had an inhibitory effect on host cell responses mediated by signaling motifs of the C terminus. CagA 200–1216 displayed a significantly greater effect on host cell elongation and constriction of the apical surface area compared with wild type CagA. The concept that CagA function is regulated by the interplay of two membrane-targeting domains is emphasized by the observation made with

a CagA Δ 200–800 mutant, where CagA 1–200 tethers CagA 800–1216 to the membrane compartment. The effect of CagA 800–1216 on cell elongation is significantly reduced in CagA Δ 200–800. Disruption of the N-terminal membrane-targeting domain (CagA 1–200) through the deletion of the first 26 AA abolishes its inhibitory effects on C terminus-mediated changes in host cell morphology. The covalent bond between CagA 1–200 and the C terminus is essential for its inhibitory effect, because when expressed in *trans*, CagA 1–200 loses its inhibitory function on CagA 200–1216. Together these data suggest that tethering of signaling motifs in the C terminus of CagA via the N terminus domain 1–200 to a different membrane substructure influences host cell responses.

Whereas CagA 1–200 does not have an effect on cell elongation or apical surface constriction on its own, we could show that it increases the formation of cell-cell adhesion. This observation is contrary to the well established fact that CagA induces cell scattering in subconfluent cells (14, 15, 23, 48). Using a cell-cell adhesion assay, we eliminated the influence of cell-extracellular matrix interaction, because cell-cell and cell-extracellular matrix adhesion are interdependent processes (49, 50). In this setting, CagA WT-expressing cells are not different in rate of cell-cell contact formation or in strength of formed contacts compared with control cells. The C terminus membrane-targeting domain CagA 200–1216 causes the loss of cell-cell adhesion as it decreases the rate of formation and the strength of newly formed cell-cell contacts significantly. This is counteracted by a considerable increase of cell-cell adhesion through the CagA N terminus membrane-targeting domain 1–200. The observed difference to subconfluent cells attaching to a surface area, where CagA WT induces cell scattering, could be due to increased cell motility, hence changes in cell-extracellular matrix interaction exerting additional effects on cell-cell adhesion (47, 50). However, in a complex network of epithelial cells in gastric tissue *in vivo*, where CagA-induced cell scattering has not been observed, the direct effect of CagA on cell-cell adhesion may play a bigger role than in subconfluent cells *in vitro*.

The impact of CagA on cell-cell adhesion has been linked to its ability to stimulate TCF/ β -catenin transcriptional activity. Increased TCF/ β -catenin transcriptional activity is associated with cancer formation, and it has been suggested that CagA may exert its carcinogenic effects through this pathway (51). The current understanding is that CagA destabilizes the E-cadherin/ β -catenin complex at cell-cell junctions, which causes loss of cell-cell adhesion and increase of cytoplasmic β -catenin (17, 34, 36). The CagA membrane-targeting domain 1–200 co-localizes with β -catenin by immunofluorescence at the lateral membrane of newly formed cell-cell contact sites in subconfluent epithelial cells, and it increases the formation of cell-cell adhesion. These functional data suggest that CagA 1–200 stabilizes the cadherin/catenin protein complex. Consistent with this finding is that CagA 1–200 covalently bound to CagA 200–1216 decreases TCF/ β -catenin transcriptional activity, which is induced by the C-terminal CagA membrane-targeting domain alone.

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Although this study did not test the role of the bacterial effector protein CagA for carcinogenesis, our data imply that the CagA membrane-targeting domain 1–200 could be an intrinsic inhibitor of the carcinogenic potential of CagA. Therefore, bacterial or host factors that would alter the inhibitory effect of the N-terminal membrane-targeting domain of CagA could have an impact on gastric carcinogenesis.

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