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Helicobacter pylori cytotoxin-associated gene A (CagA) subverts the apoptosis-stimulating protein of p53 (ASPP2) tumor suppressor pathway of the host

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Contributed by Rino Rappuoli, April 19, 2011 (sent for review February 8, 2011)

Type I strains of Helicobacter pylori (Hp) possess a pathogenicity island, cag, that encodes the effector protein cytotoxin-associated gene A (CagA) and a type four secretion system. After translocation into the host cell, CagA affects cell shape, increases cell motility, abrogates junctional activity, and promotes an epithelial to mesenchymal transition-like phenotype. Transgenic expression of CagA enhances gastrointestinal and intestinal carcinomas as well as myeloid and B-cell lymphomas in mice, but the mechanism of the induced cancer formation is not fully understood. Here, we show that CagA subverts the tumor suppressor function of apoptosis-stimulating protein of p53 (ASPP2). Delivery of CagA inside the host results in its association with ASPP2. After this interaction, ASPP2 recruits its natural target p53 and inhibits its apoptotic function. CagA leads to enhanced degradation of p53 and thereby, down-regulates its activity in an ASPP2-dependent manner. Finally, Hp-infected cells treated with the p53-activating drug Doxorubicin are more resistant to apoptosis than uninfected cells, an effect that requires ASPP2. The interaction between CagA and ASPP2 and the consequent degradation of p53 are examples of a bacterial protein that subverts the p53 tumor suppressor pathway in a manner similar to DNA tumor viruses. This finding may contribute to the understanding of the increased risk of gastric cancer in patients infected with Hp CagA + strains.

Results

CagA Associates with the Tumor Suppressor ASPP2. To investigate the role of the individual domains of CagA, we used an in vivo biotinylation approach combined with affinity purification to identify interacting proteins that selectively bind one of two domains in addition to the full-length protein (Fig. SLA). A biotin acceptor peptide (AP) followed by a tobacco etch virus (TEV) cleavage site was placed N-terminally of full-length (AP-CagA), N terminus (AP-Nt CagA), or C terminus (AP-Ct CagA) of CagA. We included a biotin-resistant version of the acceptor peptide fused to full-length CagA (AP + CagA) (Fig. SLA) as a negative control. The fusion constructs were coexpressed in a doxycycline-inducible manner in T-REx293 cells together with the biotin ligase BirA, which biotinylates the AP tag as revealed by streptavidin HRP immunoblotting (Fig. 1A). We chose this strategy to provide an affinity handle at the extremity of the constructs, while allowing for proper integration into possible junctional complexes of the cell remain intact, and the cells do not become migratory or invasive.

In contrast to the C terminus of CagA, few proteins were found to interact with its N terminus. In the absence of the N-terminal domain, the C terminus of CagA is primarily localized to the cytoplasm. Coexpression of the C- and N-terminal domains induces a strong accumulation of both domains near the plasma membrane. Thus, the main role ascribed to the N terminus is to target CagA to the plasma membrane (7).

Earlier reports have linked injection or exogenous expression of CagA to the activation of several oncogenic pathways. For example, destabilization of the E-Cadherin/β-catenin complex by CagA induces abnormal activation of the wingless/int (WNT)/β-catenin pathway (17, 18). However, the effects of CagA on tumor suppressor pathways have remained obscure. Using a proteomic approach, we here show that the N terminus of CagA interacts with the tumor suppressor apoptosis-stimulating protein of p53 (ASPP2). ASPP2 is a proapoptotic protein that associates with and activates the tumor suppressor p53 on DNA damage or oncogenic stimuli, thereby inducing apoptosis. We show that, after binding to CagA, ASPP2 binds its natural ligand p53. However, the interaction between ASPP2 and p53 results in proteosomal degradation of p53 and consequently, inhibition of the apoptotic response of the host cell. CagA, thus, hijacks ASPP2 and alters its activity in a manner that promotes cell survival and favors transformation.
CagA interacts with the tumor suppressor ASPP2. (A) Exogenous expression of BirA induces biotinylation of the AP-tagged CagA constructs. T-REx 293 stable transfectants were treated overnight with Dox to induce the expression of the indicated constructs together with the birin ligase BirA. Total cell lysates were immunoblotted (IB) with streptavidin-HRP (Upper) and anti-CagA antibody (Lower). Full-length AP-CagA is partially processed into two fragments. Both full-length and N-terminal fragments are recognized by streptavidin-HRP. *Non-specific band recognized by streptavidin-HRP. (B) AP-NT CagA associates with endogenous ASPP2. Amino acid sequence of ASPP2. Peptides highlighted in bold were identified by LC-MS/MS in T-REx 293 expressing AP-NT CagA and BirA. (C) Exogenously expressed CagA binds ASPP2 through its N-terminal domain. T-REx 293 stable transfectants were treated overnight with Dox to induce the expression of the indicated constructs together with BirA. Biotinylated proteins were retrieved by immunoprecipitation (IP) with streptavidin-conjugated beads. Total cell lysate (TCL) and IP were IB with the indicated antibodies. (D) HEK293T cells were cotransfected with FLAG-ASPP2 and AP-CagA. CagA was retrieved by IB with anti-CagA antibody, and immunoprecipitates were IB with the indicated antibodies.

Complexes with host proteins with otherwise minimal perturbations. After overnight induction with doxycycline, [35S] methionine/cysteine-labeled cells were lysed, and biotinylated proteins were recovered by absorption onto streptavidin-conjugated beads. The material was treated with TEV protease to release CagA and any proteins associated with it, which were then analyzed by SDS-PAGE and autoradiography. We observed a prominent band of 150 kDa unique to samples from cells expressing full-length CagA or its N-terminal domain (Fig. 1B). On a preparative scale, we identified by liquid chromatography LC coupled with tandem MS (LC-MS/MS) several CagA-interacting proteins previously linked to its localization or activity, including Cadherin and a member of the partitioning defective complex protein (Par-3) (Fig. S1 B and D). In addition, LC-MS/MS analysis of the 150 kDa polypeptide unambiguously identified, with excellent sequence coverage, ASPP2, which interacted with full-length CagA and its N terminus (Fig. 1 B and C and Fig. S1C) but not with its C terminus (Fig. 1C).

Immunoprecipitation of FLAG-ASPP2 from cells coexpressing AP-CagA confirmed the interaction between ASPP2 and CagA (Fig. 1D).

CagA Relocalizes ASPP2 on Hp Infection. ASPP2 is part of the ASPP family, which includes the proapoptotic ASPP1 and the anti-apoptotic ASPP2. Both ASPP1 and ASPP2 are known activators of the tumor suppressor p53 in response to DNA damage or oncogenic stimuli (19). The C terminus of ASPP2 transiently interacts with the DNA binding domain of p53, and after this interaction, p53 induces the expression of genes involved in apoptosis, although the molecular mechanism used by ASPP2 to prime p53 to the apoptotic response is still unclear (20, 21). ASPP2 also binds other factors with roles in apoptosis (Bcl-2 and Yes-associated protein) (22–24) and control of cell growth (p65/RelA subunit of NF-kB, adenomatous polyposis coli tumor suppressor, and protein phosphatase-1) (24–26).

To place this newly identified interaction in the context of infection with Hp, we infected the gastric adenocarcinoma cell line (AGS), bearing WT p53, with WT Hp (G27) or the isogenic HpΔVirB10, which lacks the secretion machinery required for CagA injection. Surprisingly, we were unable to detect ASPP2 in cells infected with WT Hp when lysis was performed using a mild, Brij-containing buffer (Fig. 2A). However, Hp-infected cells lysed in an SDS-based buffer showed a slight increase in total ASPP2 levels compared with cells infected with either ΔVirB10 or ΔCagA Hp mutants (Fig. S2A). These results suggest that Hp induces relocalization rather than degradation of ASPP2.

To further explore this possibility, AGS cells were infected with Hp or the isogenic mutants ΔVirB10 or ΔCagA and lysed in 0.5% Triton X-100. As observed before, the amount of endogenous ASPP2 recovered was reduced in samples exposed to WT Hp (Fig. 2B). However, resolubilization of the Triton-resistant pellet in SDS lysis buffer confirmed that ASPP2 was relocalized to a Triton-resistant fraction, which contains cell remnants, nuclei, Triton-resistant membranes, and bacteria (Fig. 2B).

To determine whether CagA affects the localization of ASPP2, we transfected AGS cells with Cherry-ASPP2 alone or in combination with GFP-CagA. Cherry-ASPP2 is present in the cytoplasm and is excluded from the nucleus. Coexpression of Cherry-ASPP2 with GFP-CagA shows strong colocalization, and CagA recruits ASPP2 to a region close to the plasma membrane (Fig. 2C and Fig. S2B). Although there is no clear consensus as to the exact localization of ASPP2 (27, 28), a recent study described the targeting of ASPP2 to tight junctions (29). Because full-length CagA is also found close to the plasma membrane and junctional complexes, CagA likely recruits ASPP2 to form a complex near the plasma membrane.

To confirm the interaction between CagA and ASPP2 on infection, we immunoprecipitated CagA from Hp-infected AGS cells that were lysed by sonication. ASPP2 immunoblotting confirmed the CagA-ASPP2 interaction in the course of infection with WT but not ΔVirB10 Hp (Fig. 2D). Thus, we confirmed that CagA equally targets ASPP2 in transfectants and on in vivo infection, and it relocalizes ASPP2 to a region near the plasma membrane.

ASPP2 Forms a Complex with p53 on Delivery of CagA. To determine whether the binding of CagA to ASPP2 and the subsequent redistribution of ASPP2 affects the interaction between ASPP2 and p53, AGS cells were infected with WT or ΔVirB10 mutant Hp, and the cytoplasmic fraction of endogenous p53 was immunoprecipitated. In the absence of infection, ASPP2 does not bind p53 (Fig. 3A). However, in cells exposed to WT Hp, we observed an association between endogenous p53 and ASPP2. This interaction requires CagA, because it was not observed in cells infected with the ΔVirB10 mutant Hp (Fig. 3A). Thus, injection of CagA facilitates the interaction between p53 and ASPP2. Of note, treatment of cells with Doxorubicin (Dox), a DNA damaging agent that activates p53, induces the association of ASPP2 with p53 as well as apoptosis in a concentration-dependent manner.
dependent manner (Fig. S3 A and B) without affecting the cellular distribution of ASPP2 (compared with cells infected with WT Hp) (Fig. S3C).

To exclude possible competition between CagA and p53 for binding to ASPP2, we mapped the region of ASPP2 required for binding to CagA. We cotransfected FLAG-tagged fragments of ASPP2 with AP-CagA in HEK293T cells (Fig. S4A). Only full-length ASPP2 and its 861aa N-terminal region, but not its predicted N-terminal coiled coil domain or C terminus, were recovered together with AP-CagA (Fig. S4B). Thus, although p53 binds ASPP2 at its C terminus, CagA binds at its N terminus and thus, is unlikely to compete with p53.

To understand the sequence of events triggered by the translocation of CagA, we determined the kinetics between CagA and ASPP2, and ASPP2-p53. AGS cells were infected with WT Hp or ΔVirb10 mutant and harvested at different time points. We detected an association between CagA and ASPP2 already 90 min postinfection (p.i.), with a additional increase as time progressed (Fig. 3B and Fig. S5A). In contrast, the association between ASPP2 and cytoplasmic p53 was observed not earlier than 3 h p.i. and reached a peak 7 h p.i. This suggests that ASPP2 recruits the cytoplasmic pool of p53 after ASPP2 has been engaged by CagA. We did not detect a ternary complex composed of CagA, ASPP2, and p53. Thus, CagA modulates both the localization of ASPP2 as well as its interaction with the tumor suppressor p53.

To determine whether the interaction between ASPP2 and p53 involves phosphorylation of CagA and initiation of RTK signaling, we infected AGS cells with the mutant Hp EPISA, which translates a phosphorylation-resistant version of CagA. In cells infected with Hp-EPISA, p53 binds ASPP2 to the same extent as seen for WT Hp (Fig. S5B). Thus, CagA stimulates the association between ASPP2 and p53 in a phosphorylation-independent manner.

CagA Stimulates Proteasomal Degradation of p53 in an ASPP2-Dependent Manner. p53 is a transcription factor that regulates a variety of target genes. It is usually rapidly degraded by the proteasome but stabilized on DNA damage or cellular stress. Because ASPP2 recruits cytoplasmic p53 on Hp infection, we hypothesized that the transcriptional activity of p53 might be altered on translocation of CagA. We examined the levels and activity of p53 in response to DNA damage (Dox) in cells previously infected with Hp. Treatment of cells with Dox elicited a drastic increase in p53 levels, and as a consequence, its downstream effectors p21 and Bax were up-regulated (Fig. 4A). However, Dox treatment of cells infected with WT or the EPISA Hp mutant inhibited accumulation of p53 (Fig. 4A). Accordingly, a decrease in p53 also inhibited its transcriptional activity; Bax and p21 are not up-regulated in Dox-treated and Hp-infected cells. The failure to up-regulate p21 and Bax is a direct consequence of reduced levels of p53, because infection of the p53-deficient isogenic cell line with Hp or Hp ΔVirb10 mutant followed by Dox treatment did not affect Bax or p21 levels (Fig. S6B). WT Hp and the EPISA mutant affect p53 levels equally. Phosphorylation of CagA is, therefore, not required for inhibition of the p53 response and suggests a role for the phosphorylation-independent interaction between CagA and ASPP2.

To investigate whether the failure to up-regulate p53 indeed requires ASPP2, we expressed the FLAG-tagged N-terminal segment of ASPP2 (FLAG 861-aa ASPP2) followed by Hp infection and Dox treatment. This N-terminal part of ASPP2 binds CagA, but the absence of the ankyrin (ANK), proline-rich, and SH3 domains prevents the binding to p53 (Fig. S4 C and D). Thus, this ASPP2 fragment is expected to compete with endogenous ASPP2 for binding to CagA and therefore, prevent the downstream events. Indeed, p53 accumulated normally on Dox treatment of cells infected with Hp and expressing the N-terminal 861-aa fragment of ASPP2, whereas in Hp-infected control cells (expressing FLAG 330-aa ASPP2), up-regulation of p53 was
inhibited (Fig. 4B). Thus, CagA-mediated inhibition of p53 expression is dependent on ASPP2.

To test whether CagA targets p53 for degradation by the proteasome, cells were treated with Dox and subsequently incubated with the proteasome inhibitor ZL3VS. This caused accumulation of p53 in uninfected as well as the Virb10 Hp mutant-infected cells (Fig. 4C). Inhibition of p53 degradation was effective as well in cells infected with WT Hp and treated with Dox, indicating that CagA-induced degradation of p53 is mediated by the proteasome (Fig. 4C).

**Down-Regulation of ASPP2 Promotes the Apoptotic Response on Hp Infection.** Under normal conditions, the tumor suppressor function of the ASPP2-p53 pathway is exerted mainly through induction of the apoptotic response. However, in Hp-infected cells, CagA promotes the interaction between p53 and ASPP2, leading to an enhanced degradation of p53 and therefore, inhibition of its transcriptional activity. Hp induces only a slight increase in the apoptotic response of infected cells, and apoptosis is inhibited by the delivery of CagA, which also activates a prosurvival pathway that stabilizes and enhances the antiapoptotic activity. Hp induces only a slight increase in the transcriptional activity of p53, and apoptosis is inhibited by the delivery of CagA, which also activates a prosurvival pathway that stabilizes and enhances the antiapoptotic activity. Hp induces only a slight increase in the transcriptional activity of p53, and apoptosis is inhibited by the delivery of CagA, which also activates a prosurvival pathway that stabilizes and enhances the antiapoptotic activity.
CagA inhibits p53 accumulation and transcriptional activity in an ASPP2-dependent manner. (A) CagA inhibits the accumulation and transcriptional activity of p53. HCT116 p53<sup>−/−</sup> cells were infected for 24 h with the indicated Hp strains (moi = 1:50) or left uninfected. Where indicated, cells were treated 5 h postinfection with 1 μg/mL Dox for 1.5 h. SDS-TCLs were IB with the indicated antibodies. p97 serves as loading control. We used HCT116 p53<sup>−/−</sup> cells, because these can be infected with Hp (SI Materials and Methods and Fig. S6A) and their p53 pathway is well-characterized (41). (B) CagA inhibits accumulation of p53 in an ASPP2-dependent manner. HCT116 stably transfected with the indicated constructs were treated as indicated in A. To compare the level of endogenous ASPP2 with the overexpressed constructs, SDS lysates were IP and IB with ASPP2 antibody. This experiment is representative of three independent experiments. FLAG 330-aa ASPP2 construct is not shown, because the ASPP2 antibody does not recognize this short fragment (SI Materials and Methods and Fig. S4A). (C) CagA induces proteasomal degradation of p53. HCT116 cells were infected for 10 h with the indicated Hp strains (1:100 moi) or left uninfected. Where indicated, cells were treated 3 h postinfection with 1 μg/mL Dox for 1 h. Where indicated, ZL<sub>V5</sub> was added for the remaining hours before harvesting the cells. TCLs were IB with the indicated antibodies. (D) ASPP2 inhibits apoptosis on Hp infection. Levels of cleaved Caspase-3 (CC3) assayed by flow cytometry in shGFP (white bars) or shASPP2 (construct #3; black bars) AGS cells that were infected for 24 h with the indicated Hp strains (1:50 moi) or left uninfected. Error bars ± SEM (n = 5). *P ≤ 0.05; **P < 0.01; ***P < 0.001. Significance was tested using two-way ANOVA Bonferroni multiple-comparison test. (E) Dox-induced apoptosis is inhibited by Hp in an ASPP2-dependent manner. Relative increase in CC3 in shGFP (white bars) or shASPP2 (construct #3; black bar) cells infected for 24 h with the indicated Hp strains (1:50 moi) or left uninfected and treated with 1 μg/mL Dox for 1 h. Error bars ± SEM (n = 3). *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001. Significance was tested using two-way ANOVA Bonferroni multiple-comparison test.

Fig. 4. CagA inhibits p53 accumulation and transcriptional activity in an ASPP2-dependent manner. (A) CagA inhibits the accumulation and transcriptional activity of p53. HCT116 p53<sup>−/−</sup> cells were infected for 24 h with the indicated Hp strains (moi = 1:50) or left uninfected. Where indicated, cells were treated 5 h postinfection with 1 μg/mL Dox for 1.5 h. SDS-TCLs were IB with the indicated antibodies. p97 serves as loading control. We used HCT116 p53<sup>−/−</sup> cells, because these can be infected with Hp (SI Materials and Methods and Fig. S6A) and their p53 pathway is well-characterized (41). (B) CagA inhibits accumulation of p53 in an ASPP2-dependent manner. HCT116 stably transfected with the indicated constructs were treated as indicated in A. To compare the level of endogenous ASPP2 with the overexpressed constructs, SDS lysates were IP and IB with ASPP2 antibody. This experiment is representative of three independent experiments. FLAG 330-aa ASPP2 construct is not shown, because the ASPP2 antibody does not recognize this short fragment (SI Materials and Methods and Fig. S4A). (C) CagA induces proteasomal degradation of p53. HCT116 cells were infected for 10 h with the indicated Hp strains (1:100 moi) or left uninfected. Where indicated, cells were treated 3 h postinfection with 1 μg/mL Dox for 1 h. Where indicated, ZL<sub>V5</sub> was added for the remaining hours before harvesting the cells. TCLs were IB with the indicated antibodies. (D) ASPP2 inhibits apoptosis on Hp infection. Levels of cleaved Caspase-3 (CC3) assayed by flow cytometry in shGFP (white bars) or shASPP2 (construct #3; black bars) AGS cells that were infected for 24 h with the indicated Hp strains (1:50 moi) or left uninfected. Error bars ± SEM (n = 5). *P ≤ 0.05; **P < 0.01; ***P < 0.001. Significance was tested using two-way ANOVA Bonferroni multiple-comparison test. (E) Dox-induced apoptosis is inhibited by Hp in an ASPP2-dependent manner. Relative increase in CC3 in shGFP (white bars) or shASPP2 (construct #3; black bar) cells infected for 24 h with the indicated Hp strains (1:50 moi) or left uninfected and treated with 1 μg/mL Dox for 1 h. Error bars ± SEM (n = 3). *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001. Significance was tested using two-way ANOVA Bonferroni multiple-comparison test.
sonication in Nonidet P-40-containing buffer (0.05% Nonidet P-40, 20 mM Hepes, pH 7.8, 50 mM KCl, 5 mM EDTA, 5% Glycerol) supplemented with complete protease inhibitors (Roche) and cleared by centrifugation. To detect endogenous p53/ASPP2 interaction during the course of Dox treatment, cells were lysed by sonication in 20 mM Hepes (pH 7.8), 400 mM KCl, 5% Glycerol, 2 mM EDTA, and 0.5% NP-40 supplemented with complete protease inhibitors (Roche) and cleared by centrifugation. Detailed description of the immunoprecipitation is in SI Materials and Methods and Table S1.

Flow Cytometry. Cells were stained with Cleaved Caspase 3 antibody according to the manufacturer's instructions. Cells were analyzed using a Becton Dickinson FACs-Calibur, and apoptotic profiles were generated using Flowjo 8.5.3 software.

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