Helicobacter pylori secretes an 88-kDa vacuolating cytotoxin (VacA) that may contribute to the pathogenesis of peptic ulcer disease and gastric cancer. VacA cytotoxic activity requires assembly of VacA monomers into oligomeric structures, formation of anion-selective membrane channels, and entry of VacA into host cells. In this study, we analyzed the functional properties of recombinant VacA fragments corresponding to two putative VacA domains (designated p33 and p55). Immunoprecipitation experiments indicated that these two domains can interact with each other to form protein complexes. In comparison to the individual VacA domains, a mixture of the p33 and p55 proteins exhibited markedly enhanced binding to the plasma membrane of mammalian cells. Furthermore, internalization of the VacA domains was detected when cells were incubated with the p33/p55 mixture but not when the p33 and p55 proteins were tested individually. Incubation of cells with the p33/p55 mixture resulted in cell vacuolation, whereas the individual domains lacked detectable cytotoxic activity. Interestingly, sequential addition of p55 followed by p33 resulted in VacA internalization and cell vacuolation, whereas sequential addition in the reverse order was ineffective. These results indicate that both the p33 and p55 domains contribute to the binding and internalization of VacA and that both domains are required for vacuolating cytotoxic activity. Reconstitution of toxin activity from two separate domains, as described here for VacA, has rarely been described for pore-forming bacterial toxins, which suggests that VacA is a pore-forming toxin with unique structural properties.

Functional Properties of the p33 and p55 Domains of the Helicobacter pylori Vacuolating Cytotoxin*

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Victor J. Torres‡§, Susan E. Ivie‡, Mark S. McClain¶, and Timothy L. Cover†‡***

From the Departments of ‡Microbiology and Immunology and ¶Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-2605 and **Department of Veterans Affairs Medical Center, Nashville, Tennessee 37212

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§ Supported in part by the Vanderbilt University Medical Center Intramural Discovery Grant Program.
¶ To whom correspondence should be addressed: Div. of Infectious Diseases, A2200 MCN, Vanderbilt University School of Medicine, Nashville, TN 37232. Tel.: 615-322-2035; E-mail: timothy.L.cover@vanderbilt.edu.

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internalization into cells and reconstitution of vacuolating cytotoxic activity.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions—Escherichia coli** DH5α was used for plasmid propagation and was grown in Luria-Bertani (LB) broth or on LB agar at 37 °C. For expression of recombinant proteins, expression plasmids were transformed into E. coli strain JM109 (DE3). Promega), which encodes an isopropyl-β-D-thiogalactopyranoside-inducible copy of the RNA polymerase gene from bacteriophage T7. Transformants were grown in Terrific broth (Invitrogen) supplemented with 25 μg of kanamycin/ml (TB-KAN). H. pylori wild-type strain 60190 (American Type Culture Collection 49503; Manassas, VA) and strain VT330 (encoding a c-Myc-tagged VacA protein) were grown on Trypticase soy agar plates containing 5% sheep blood at 37 °C in ambient air containing 5% CO₂. Cultures were then induced with a final isopropyl-β-D-thiogalactopyranoside (IPTG) concentration of 0.5 mM and incubated at 25 °C for 16–18 h (p55). P.37° C. The cells were then washed with two times with phosphate-buffered saline and then incubated in fetal bovine serum-free tissue culture medium, containing 10 mM ammonium chloride and gentamicin, for 4 h. For the p33/p55 complementation assays, both soluble extracts were mixed and incubated for 1 h at 25 °C prior to addition to the medium overlying the cells. Purified VacA from H. pylori culture supernatant was routinely acid-activated prior to testing in cell culture assays (40, 50), whereas E. coli soluble extracts were not acid-activated (51). After incubation, cell vacuolation was examined by inverted light microscopy and quantified by a neutral red uptake assay (52). Neutral red uptake assay data are presented as mean ± standard deviation.

**Immunoprecipitation of VacA Complexes—E. coli** soluble extracts containing different recombinant VacA proteins were normalized as described above. Normalized soluble extracts were then mixed and incubated for 1 h at 25 °C. VacA complexes were immunoprecipitated with an anti-c-Myc monoclonal antibody (2 μg/ml antibody 9E10) and protein G-coated beads (Zymed Laboratories Inc.) (48). To analyze interactions between p33, p55, and p33/p55 mixture with full-length VacA, normalized soluble extracts were mixed with acid-activated c-Myc-tagged VacA (Vac-VacA) purified from H. pylori culture supernatant (2 μg/ml) for 1 h at 25 °C, and the proteins were immunoprecipitated as described above. Immunoprecipitated proteins were analyzed by immunoblotting with an anti-His (Santa Cruz Biotechnology) or anti-c-Myc monoclonal antibody (9E10), followed by secondary antibodies conjugated with horseradish peroxidase (Bio-Rad), as described above.

**Analysis of VacA Binding and Internalization into Mammalian Cells**—To analyze interactions of VacA with the surface of cells, E. coli soluble extracts, containing recombinant VacA proteins, were added to HeLa cells grown on cover glasses in 6-well plates for 1 h at 4 °C or 37 °C. VacA interactions with mammalian cells were then analyzed by indirect immunofluorescence (12, 41). Briefly, the cells were washed with Tris-buffered saline (TBS) (10 mM Tris, 150 mM NaCl, pH 7.5) and fixed with 3.7% formaldehyde. Fixed cells were incubated with an anti-c-Myc antibody (1:500) or with an anti-VacA polyclonal antiserum (1:500) followed by secondary antibodies conjugated with horseradish peroxidase (Bio-Rad), as described above.
For immunoblot analysis of VacA interactions with cells, HeLa cells were seeded into a 96-well plate and incubated with E. coli soluble extracts, as described above. Cells were then washed three times with TBS and lysed directly in the wells of the 96-well plate by adding SDS-containing buffer. The presence of VacA in these cell lysates was detected by immunoblotting, as described above.

RESULTS

Expression of Recombinant p33 and p55 VacA Domains—To study functional properties of the p33 and p55 VacA fragments (Fig. 1A), we expressed these two putative domains as recombinant proteins using a recently developed system that allowed expression of a functional cytotoxic form of full-length VacA in E. coli (51). Soluble E. coli extracts containing the recombinant p33 and p55 domains were generated as described under "Experimental Procedures." Both the recombinant p33 and p55 proteins were successfully expressed based on immunoblotting analysis (Fig. 1B).

p33 and p55 VacA Domains Complement Each Other for Vacuolating Activity—Previously, it has been shown that E. coli soluble extract containing the full-length 88-kDa recombinant VacA protein exhibits vacuolating cytotoxic activity when added to mammalian cells (51). Therefore, we investigated whether either the p33 or the p55 protein was capable of causing cell vacuolation activity when added to mammalian cells. No detectable vacuolating activity was observed when E. coli extracts containing the p33 or p55 protein were added individually to HeLa cells (Fig. 2A). In contrast, when extracts containing the p33 and p55 proteins were mixed and then added to HeLa cells, extensive cell vacuolation was detected, based on results of a neutral red uptake assay and also based on light microscopic examination (Fig. 2A and data not shown). Extensive vacuolating activity was also observed when gastric cell lines (AGS and AZ-521) were intoxicated with the p33/p55 mixture (data not shown). As seen with purified VacA, the vacuolating activity exhibited by the mixture of the recombinant p33 and p55 VacA domains was dose-dependent (Fig. 2B).

These results indicate that a mixture of recombinant p33 and p55 VacA domains is capable of reconstituting vacuolating cytotoxic activity.

p33 and p55 Domains Form Oligomeric Complexes—We hypothesized that the ability of the p33 and p55 proteins to complement each other for vacuolating activity might require the formation of protein complexes comprising these two proteins. To test whether the p33 and p55 VacA domains could physically interact, we mixed different combinations of these epitope-tagged recombinant proteins and then performed a series of immunoprecipitation experiments using an anti-c-Myc antibody, as described under "Experimental Procedures." In these experiments, p33/p55 interactions were detected (Fig. 3A and lane 1). However, we were unable to detect the assembly of p33 and p55 domains into large 1,000-kDa complexes similar to those formed by the 88-kDa secreted VacA.
protein present in *H. pylori* broth culture supernatant, based on analysis involving gel filtration chromatography (data not shown).

**Interactions of p33 and p55 Domains with Full-length 88-kDa VacA**—We next investigated whether the recombinant p33 and p55 proteins could physically interact with the wild-type 88-kDa VacA protein purified from *H. pylori* culture supernatant. For these studies, we used a c-Myc-tagged 88-kDa VacA protein (Myc-VacA) purified from *H. pylori* culture supernatant (2 μg/ml), and proteins were immunoprecipitated with an anti-c-Myc antibody. Immunoprecipitated proteins were analyzed by immunoblotting as described above, with an anti-His antibody (top panels) or anti-c-Myc antibody (bottom panel).

**Intracellular Localization of the p33 and p55 VacA Domains**—We next investigated whether the p33 and p55 proteins were internalized into mammalian cells. HeLa cells were intoxicated with either purified VacA from *H. pylori* or *E. coli* soluble extracts containing the p33 domain, the p55 domain, or the p33/p55 mixture, as described under “Experimental Procedures.” Internalized VacA was visualized by indirect immunofluorescence analysis of permeabilized cells. As expected, the 88-kDa VacA purified from *H. pylori* was internalized into HeLa cells (Fig. 5, panels 1 and 3). Little if any internalization of the p33 or p55 protein was detected when these proteins were added individually to cells (Fig. 5, panels 2 and 3). In contrast, when the p33 and the p55 proteins were mixed and then added to HeLa cells, both proteins were detected on the surface of HeLa cells (Fig. 4B, panels 4 and 8). Thus, binding of p55 to the cell surface was detected by immunofluorescence assay when the p33 and p55 domains were added together to cells, but not when p55 was added independently to cells. Interestingly, the distribution of p33 on the surface of HeLa cells was punctate when p33 was added alone to cells, whereas it was continuous (non-punctate) when p33 was added to cells together with the p55 domain (Fig. 4B, panels 3 and 4). These data indicate that the interactions of p33 and p55 VacA domains with the surface of cells are substantially altered when both domains are present.

**Sequential Addition of p55 and p33 VacA Domains**—To further investigate the role of p33 and p55 interactions with host cells, we sequentially incubated HeLa cells with *E. coli* soluble extract containing p33 followed by extract containing p55 (p33<sup>1</sup>, p55<sup>2</sup>), or p55 followed by p33 (p55<sup>1</sup>, p33<sup>2</sup>) (superscript numbers indicate the order in which the samples were added to the cells) (Fig. 6). As in all of the preceding experiments, the concentrations of p33 and p55 proteins added to the cells were not shown), or when cell binding was assessed using gastric cell lines (AGS and AZ-521) instead of HeLa cells (data not shown). As a second approach to investigate p33 and p55 interactions with mammalian cells, we used indirect immunofluorescence methodology. As expected, full-length 88-kDa c-Myc-tagged VacA purified from *H. pylori* bound to the surface of HeLa cells, and the binding could be detected with either an anti-c-Myc antibody or a polyclonal anti-VacA antiserum that recognizes the p55 domain (Fig. 4B, panels 1 and 5) (12, 39). In contrast, no immunoreactive signal on the surface of cells was detected with these antibodies following incubation of cells with negative control extracts (Fig. 4B, pET, panels 2 and 6). When recombinant p33 and p55 domains were added individually to cells, binding of p33 (either p33Myc-His or p33His) to the surface of the cells was detectable (Fig. 4B, panel 3, and data not shown), but binding of p55 was not detected (Fig. 4B, panel 7). As shown in Fig. 3A, we were able to detect binding of the p55 domain to the surface of cells by immunoblot methodology, but we were unable to detect interaction of the recombinant p55 protein with the surface of HeLa cells using immunofluorescence assays, despite testing two different forms of this protein (p55His or p55Myc) and multiple antibodies, including the anti-VacA polyclonal antiserum used in panel A. We presume that the relevant epitopes are not accessible to the antibodies under the conditions of the immunofluorescence assay. When the p33 and the p55 proteins were mixed and then added to HeLa cells, both proteins were detected on the surface of HeLa cells (Fig. 4B, panels 4 and 8). Thus, binding of p55 to the cell surface was detected by immunofluorescence assay when the p33 and p55 domains were added together to cells, but not when p55 was added independently to cells. Interestingly, the distribution of p33 on the surface of HeLa cells was punctate when p33 was added alone to cells, whereas it was continuous (non-punctate) when p33 was added to cells together with the p55 domain (Fig. 4B, panels 3 and 4). These data indicate that the interactions of p33 and p55 VacA domains with the surface of cells are substantially altered when both domains are present.
The capacity of the VacA proteins to interact with the cell membrane of host cells was assessed by indirect immunofluorescence (I.F.) using anti-c-Myc (panels 1–4) and an anti-VacA polyclonal antibody that recognizes the p55 domain (panels 5–8).

FIG. 4. Binding of p33 and p55 VacA domains to mammalian cells. A. E. coli soluble extracts containing similar amounts of the indicated VacA proteins were added to HeLa cells for 1 h at 4 °C. The ability of the VacA proteins to interact with host cell membranes was assessed by immunoblot (I.B.) analysis using an anti-VacA polyclonal antibody to detect p55His and anti-His antibody to detect p33Myc-His. The anti-VacA polyclonal antibody cross-reacts with an unidentified HeLa cell protein, as indicated. B. HeLa cells were intoxicated for 1 h at 37 °C with acid-activated c-Myc-VacA (Myc-VacA; 5 μg/ml) purified from H. pylori culture supernatant (panels 1 and 5), E. coli negative control extract without VacA proteins (pET; panels 2 and 6), E. coli soluble extracts containing p33Myc-His (panel 2), p55His (panel 7), or the p33Myc-His/p55His mixture (panels 4 and 8). The capacity of the VacA proteins to interact with the cell membrane of host cells was assessed by indirect immunofluorescence (I.F.) using anti-c-Myc (panels 1–4) and an anti-VacA polyclonal antibody to detect p33Myc-His. The anti-VacA polyclonal antibody cross-reacts with an unidentified HeLa cell protein, as indicated.

FIG. 5. Internalization of p33 and p55 VacA domains into mammalian cells. Wild-type acid-activated VacA purified from H. pylori culture supernatant (panel 1) or E. coli soluble extracts containing p33Myc-His (panel 2), p55His (panel 3), or the p33Myc-His/p55His mixture (panels 4 and 5) were added to HeLa cells for 1.5 h at 37 °C. The cells were then incubated in fresh medium for an additional 16 h at 37 °C. The ability of VacA to enter into cells was assessed by indirect immunofluorescence (I.F.) of permeabilized cells using an anti-VacA antibody to detect the p55 domain (panels 1, 3, and 5) and an anti-c-Myc antibody to detect the p33Myc-His protein (panels 2 and 4).

approximately equivalent. Following incubation of cells with the first VacA protein, the culture medium overlying the cells (containing unbound VacA fragments) was washed away prior to the addition of the second VacA protein. No vacuolating activity was detected when the p33 domain was added first, followed by the p55 domain (Fig. 6A) (p331, p552). In contrast, extensive vacuolating activity was observed when the p55 domain was added first, followed by the p33 domain (Fig. 6A) (p551, p332).

To further investigate the process by which the (p551, p332) combination induced vacuolation, we analyzed the interactions of sequentially added VacA domains with the surface of host cells. In the sequential addition experiments, the intensity of the immunoblot signals was consistently weaker for the protein added first compared with the protein added second, regardless of the order of addition (p331, p552 or p551, p332). When the p55 domain was bound first, followed by the p33 domain, a condition that resulted in cell vacuolation (p551, p332) (Fig. 6A), the amount of p33 protein bound to cell membranes was increased compared with when the p33 domain was added alone (Fig. 6B, bottom panel, lane 2 versus 5). Similar results were obtained when different epitope-tagged p33 and p55 proteins (e.g. p33Myc-His and p55Myc) were used and when gastric cell lines (AGS and AZ-521) were substituted for HeLa cells (data not shown). These data indicate that binding of p55 to cells enhances subsequent binding of p33.

We next tested the hypothesis that there might be differences in the internalization of VacA, depending on the order in which VacA domains are added to cells. When the p55 domain was added first to cells, followed by the p33 domain (p551, p332), a condition that results in cell vacuolation (Fig. 6A), internalization of the p55 protein was detected (p551, p552) (Fig. 6D). Internalization of p33 was also detected, but the intensity of the internalized p33 signal was relatively weak compared with the p55 signal (data not shown). When the p33 protein was added first followed by the p55 protein (p331, p552), a condition that fails to cause cell vacuolation (Fig. 6A), neither p55 nor p33 was detected inside cells (p331, p552) (Fig. 6D and data not shown). Thus, in these sequential addition experiments, there are marked differences in the internalization of VacA depending on the order in which VacA domains are added to cells.
by indirect immunofluorescence (described above. Thereafter, the cells were incubated in fresh culture medium for an additional 16 h at 37 °C. Entry of VacA into cells was analyzed with p33 and p55 VacA domains lack detectable vacuolating activity when secreted VacA toxin undergoes proteolytic degradation to yield two fragments (p33 and p55). It has been suggested that these two fragments represent two domains of VacA (6, 31, 42, 53), but in the absence of a high resolution VacA structure, the relevant structural features of these two putative domains remain poorly characterized. In the current study, we investigated various properties of recombinant p33 and p55 VacA domains. Although our efforts to purify functional forms of p33 and p55 recombinant proteins have thus far been unsuccessful, it has nevertheless been possible to investigate various functional properties of these domains using crude preparations of the p33 and p55 recombinant proteins.

Our data indicate that the p33 and p55 VacA domains interact in solution to form protein complexes (Fig. 3). This finding is consistent with the results of previous studies, which demonstrated that p33 and p55 proteins interact in the yeast two-hybrid system (31), as well as in HeLa cells transiently co-transfected with plasmids expressing the p33 and p55 proteins (42, 54). In addition, p33 and p55 VacA fragments remain physically associated following proteolysis of the 88-kDa VacA protein secreted by H. pylori (6, 31). Notably, in the current study, heterotypic interactions between p33 and p55 were detected, but homotypic interactions (either p33/p33 or p55/p55) were not detected (Fig. 3A). This finding is consistent with the results obtained in a yeast two-hybrid system (31). Although homotypic interactions (p33/p33 or p55/p55) have not been detected in either the yeast two-hybrid system or in the current study, it remains possible that such interactions could occur in the context of a membrane environment (44) or within certain variant forms of VacA oligomers (45).

VacA 88-kDa monomers produced by H. pylori can assemble into large water-soluble oligomeric structures comprising 6–14 subunits (28–30). Our data indicate that when mixed together, p33 and p55 domains can form complexes composed of at least three independent subunits (Fig. 3B). However, we were unable to demonstrate assembly of p33 and p55 domains into high molecular mass oligomeric complexes similar to those formed by H. pylori 88-kDa monomers. Moreover, we were not able to detect interactions of either the individual p33 domain or p55 domain with the full-length secreted VacA protein from H. pylori (Fig. 3C). In contrast, when the p33/p55 mixture was combined with full-length VacA, interactions of both the p33 and p55 domains with full-length 88kDa VacA were detected (Fig. 3C). These results suggest that formation of large VacA oligomeric structures proceeds more efficiently via interactions among full-length 88-kDa VacA monomers than via interactions among isolated p33 and p55 domains.

An important conclusion of the current study is that p33 and p55 VacA domains lack detectable vacuolating activity when added individually to cells, but when mixed, the p33 and p55 domains complement each other, resulting in reconstitution of vacuolating activity (Fig. 2).
be understood in part by examining the interactions of VacA domains with the cell surface. We demonstrated in the current study that the individual p55 and p33 domains are each capable of binding to the cell surface. This finding is consistent with results of a previous study, which indicated that p55 and p33 domains can each bind to artificial lipid membranes (55, 56). It seems likely that individual p55 and p33 domains may interact with lipids on the surface of mammalian cells.

Notably, we demonstrated that VacA interactions with the surface of cells are altered in several ways when both p33 and p55 domains are present, compared with when only a single domain is present. First, in comparison to individual p33 and p55 domains, a p33/p55 mixture binds more avidly to the cell surface. This increased binding is observed for both the p33 and p55 domains (Fig. 4A). Second, when p55 is added to cells in the absence of p33, the binding of p55 is detectable in immunofluorescence assays but not in immunoblot assays. In contrast, when a mixture of p55 and p33 domains is added to cells, the binding of p55 is detectable in both assays (Fig. 4B). This suggests that the conformation or orientation of the p55 domain on the surface of cells may be altered in the presence of the p33 domain. Finally, when added individually to cells, the p33 domain localizes in a punctate distribution on the cell surface, while when added to cells along with p55, p33 localizes in a continuous (non-punctate) distribution on the cell surface (Fig. 4B). Previous studies have reported the existence of multiple cell surface receptors for VacA secreted by H. pylori, and accordingly, it seems likely that recombinant p33/p55 complexes may bind to multiple different cell surface components (see model depicted in Fig. 7, A and B).

In the current study, we demonstrated that when added together, the p33 and p55 proteins are both internalized by host cells, whereas internalization is not detectable when the p33 or p55 domains are added individually to host cells (Fig. 5). The failure of p55 to be internalized when added independently to cells is consistent with the results of a previous study, in which a mutant VacA protein consisting mainly of the p55 domain was secreted by H. pylori and bound to the surface of host cells but was not internalized (45). We propose a model in which binding of p33/p55 VacA complexes to a specific site on the cell surface (for example, a specific receptor and/or lipid rafts) promotes VacA oligomerization. We propose that the p33/p55 oligomeric complex undergoes a conformational change to permit membrane insertion of the p33 domain and that the complex can then be internalized into the cell (Fig. 7, B-D). The capacity of internalized p33/p55 complexes to induce cell vacuolation is consistent with results of a previous study, which showed that intracellular co-expression of p33 and p55 results in vacuolating cytotoxic activity (53).

Further insight into the functional roles of p33 and p55 domains comes from studies in which these domains are added sequentially to cells. We demonstrated that binding of p55 to the cell surface, followed by the addition of p33 (p551, p332), results in cell vacuolation (Fig. 6A). This result can be explained by the formation of p55/p33 complexes on the surface of the cells. We speculate that the isolated p55 domain is able to bind specific cell-surface components that promote oligomerization, membrane insertion, and internalization of VacA (Fig. 7). Thus, our model proposes that internalization of VacA into cells is dependent on an interaction of the p55 domain with specific cell surface components (Fig. 7). In support of this model, several previous studies have provided evidence indicating that amino acid sequences in the p55 domain of p88 VacA contribute to the process of VacA binding to cells (39, 45–47). In the current study, binding of p33 to the cell surface, followed by the addition of p55 (p331, p552), did not result in detectable cell vacuolation. We speculate that p33 may not be able to bind certain relevant cell surface components that are required for VacA internalization. Alternatively, sequential addition in this order (p331, p552) may not permit the formation of the p33/p55 complexes or may prevent the formation of p33/p55 complexes in the proper conformation required for internalization.

VacA causes numerous effects on intoxicated cells (7, 10, 57), and many VacA-mediated effects are dependent on the capacity of VacA to form membrane channels (11, 14, 19, 21–24). Therefore, it is of interest to view the current results in the context of what is known about functional domains of other pore-forming toxins. Two previous studies have investigated putative func-
tional domains of the pore-forming toxins aerolysin (58) and listeriolysin (59). When the two domains of aerolysin and listeriolysin were co-expressed in *Aeromonas* and *Listeria* respectively, in both cases the two domains were able to assemble into proteins with hemolytic activity (58, 59). In contrast, when the two domains of listeriolysin were expressed separately and then mixed together, no hemolytic activity was detected (59). This suggests that assembly of listeriolysin domains into a functionally active toxin may require interactions between the two domains during early stages of the protein folding process.

To the best of our knowledge, VacA is the only pore-forming toxin for which cytotoxic activity has been reconstituted from two domains during early stages of the protein folding process. Functionally active toxin may require interactions between the two domains of listeriolysin were expressed separately and then mixed together, no hemolytic activity was detected.

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