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Communication

Low pH Activates the Vacuolating Toxin of *Helicobacter pylori*, Which Becomes Acid and Pepsin Resistant*

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The protein toxin VacA, produced by cytotoxic strains of *Helicobacter pylori*, causes a vacuolar degeneration of cells, which eventually die. VacA is strongly activated by a short exposure to acidic solutions in the pH 1.5–5.5 range, followed by neutralization. Activated VacA has different CD and fluorescence spectra and a limited proteolysis fragmentation pattern from VacA kept at neutral pH. Moreover, activated VacA is resistant to pH 1.5 and to pepsin. The relevance of these findings to pathogenesis of *H. pylori*-induced gastrointestinal ulcers is discussed.

Cytotoxic strains of *Helicobacter pylori* are the major determinant in the pathogenesis of stomach and duodenal ulcers and of gastric carcinomas (1–6). Biopsies of *H. pylori*-colonized stomach epithelium show cellular swelling, expansion of endosomal compartments, and extensive vacuolation (7). *H. pylori* bacterial extracts cause a vacuolar degeneration of epithelial cells, followed by cell death in animals and in cells in culture (8–11). Vacuolar pH is acidic, as deduced from the accumulation of neutral red, a membrane-permeant amine that protonates in the vacuolar lumen (10). Determination of neutral red uptake has become the standard *in vitro* assay of *H. pylori* cytotoxicity (10, 11).

The vacuolating activity of supernatants of cytotoxic strains of *H. pylori* is due to a protein cytotoxin termed VacA[1] (12). Purified VacA causes vacuolization *in vitro* (12) and *in vivo* (13). The *vacA* gene has been recently cloned and sequenced (13–16). It encodes for a protein of 140 kDa, whose 45-kDa carboxyl-terminal portion resembles the domain responsible for translocation across the outer membrane of some bacterial proteins (17). An *H. pylori* isogenic mutant of the *vacA* gene showed no vacuolating activity (14), and VacA induces a protective immunization state in a mouse animal model (9). Purified VacA contains both a single polypeptide chain of 94 kDa and a nicked protein consisting of two fragments of 37 and 58 kDa (13). The un-nicked and nicked forms are equally cytotoxic.[2] Recently, we showed that vacuolar membranes are strongly enriched in Rab7, a small GTP binding protein largely present on late endosomal compartments (18). This finding suggests that VacA alters membrane trafficking events taking place at the late endosomal stage.

Here, we report on a remarkable property of VacA: its exposure to acidic solutions causes a very strong potentiation of vacuolating activity. Activated VacA is resistant to low pH and pepsin. VacA activation is accompanied by spectroscopic changes and by a different limited proteolysis pattern.

MATERIALS AND METHODS

VacA Preparation—H. pylori cytotoxic strain CCUG 17874 was grown as before (18). VacA was isolated either as described (12) or with a novel procedure to be described elsewhere.[3] VacA was stored as diluted solutions in phosphate buffered saline (PBS) (<0.1 mg/ml) at 4 °C. Protein concentration was measured with the Bio-Rad DC Microassay.

Assay of Cell Vacuolation—HeLa cells were cultivated and seeded as detailed before (11) in 96-well titration plates in MEM, 10% FCS at a density of $10 \times 10^3/cm^2$. Cells were incubated with VacA (9 μg/ml) in MEM, 2% FCS, 5 mM NH_4^+. To a VacA solution in PBS (1 μM), various amounts of HCl (150 mM) were added under stirring to minimize local denaturating effects, according to a previously determined titration curve. After 15 min at 37 °C (or other time periods (see Fig. 2*B*)), the solution was neutralized by addition of 150 mM NaOH under stirring or by dilution (10–20-fold) in MEM, 2% FCS, 5 mM NH_4Cl. Vacuolation was measured as described before (11).

Circular Dichroism Spectroscopy—Circular dichroism (CD) spectra were recorded on a Jasco (Tokyo, Japan) model J-710 spectropolarimeter as detailed before (19). The mean residue molecular weight for VacA residues was 110 Da, based on its sequence. CD spectra in the far-UV region (250–195 nm) were recorded in PBS, pH 7.4, at 37 °C in a 1- or 10-mm pathlength cuvette at a VacA concentration of 25 or 90 μg/ml. The protein secondary structure content was estimated with a Jasco computer program (20).

Fluorescence Spectroscopy—A Perkin-Elmer LS-50 spectrofluorimeter equipped with a 1-cm pathlength quartz cell thermostatted at 37 °C was used. VacA (32 μg/ml in PBS) fluorescence emission was monitored from 285 to 500 nm (excitation at 280 nm) and from 300 to 500 nm (excitation at 295 nm) with 5-nm slits for both excitation and emission. Spectra were averaged over four accumulations and corrected by subtracting the corresponding base line. VacA solutions were acidified and neutralized as above; the spectrum of denaturated VacA was recorded after addition of guanidinium Cl to a final concentration of 3.5 M.

Limited Proteolysis—VacA (96 μg/ml) in PBS was either kept at 37 °C or acidified to pH 2.0 with HCl, incubated for 15 min at 37 °C, and then neutralized to pH 7.4. Pronase (Boehringer, Germany) or proteinase K (Sigma) was added to a protease/substrate ratio of 1:16 and 1:50, respectively (by mass). Samples of VacA at pH 2.0 or BSA (84 μg/ml) in the same buffer were incubated with pepsin (Boehringer, Germany) at a protease/substrate ratio of 1:500 (by mass). Samples were incubated at 37 °C for 1 or 2 h, and proteolysis was stopped by adding trichloro-

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[1] The abbreviations used are: VacA, vacuolating toxin of *Helicobacter pylori*; PBS, phosphate-buffered saline; MEM, minimal essential medium; FCS, fetal calf serum; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis.

[2] M. de Bernard, E. Papini, V. de Filippis, E. Gottardi, J. Telford, R. Manetti, A. Fontana, R. Rappuoli, and C. Montecucco, unpublished results.

[3] R. Manetti, P. Massari, D. Burroni, M. de Bernard, A. Marchini, R. Olivieri, C. Montecucco, R. Rappuoli, and J. L. Telford, submitted for publication.

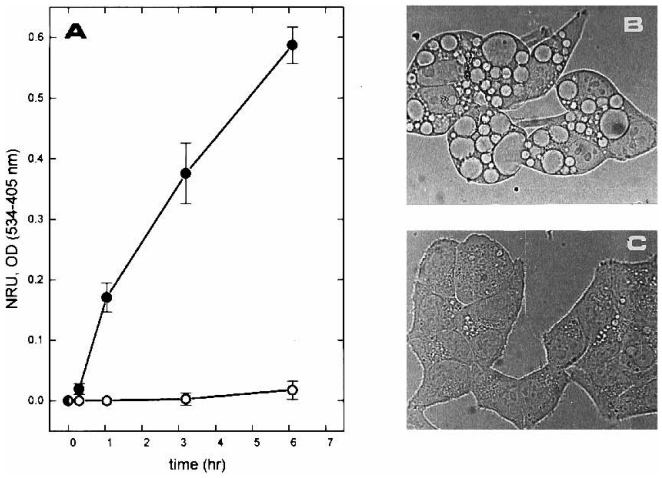

FIG. 1. **Vacuolar degeneration of HeLa cells treated with VacA preincubated at pH 7.4 or 2.0.** *A*, purified VacA (0.7 μM in PBS) was exposed for 15 min at 37 °C at pH 7.4 or 2.0 and then reneutralized to pH 7.4. HeLa cells were incubated with VacA (final concentration, 0.28 μM) for the indicated time periods, and the extent of cell vacuolization was assayed by measuring neutral red uptake (*NRU*). ●, acid-treated VacA; ○, non-acid-treated sample. Data are the average of three independent assays run in triplicate, and *bars* represent ± S.D. *B* and *C* show pictures of HeLa cells (magnification, ×145) incubated for 24 h with acid-treated and non-acid-treated VacA, respectively.

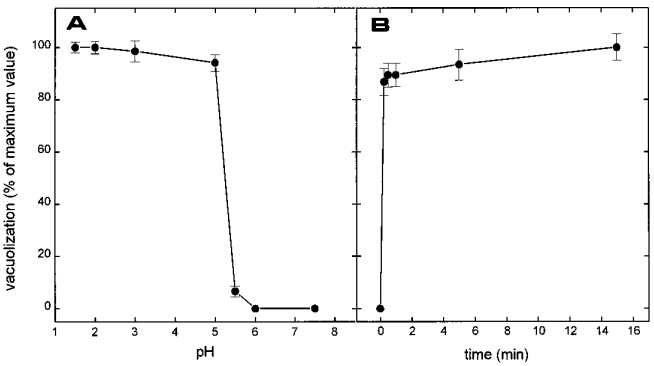

FIG. 2. **Activation of VacA by low pH.** *A*, pH dependence of the cell vacuolization activity of purified VacA (0.7 μM in PBS) exposed for 15 min at 37 °C at indicated pH values and then neutralized to pH 7.4. HeLa cells were incubated with VacA (final concentration, 70 nM) for 7 h. *B*, kinetics of the activation of VacA exposed to pH 2.0 and assayed as reported above. The extent of cell vacuolization was assayed as in the legend to Fig. 1, and data are expressed as percentages of the maximal value of dye uptake in each experiment. Points are the average of four or more experiments, and *bars* represent ±S.D.

acetic acid (final concentration, 6%). After centrifugation, samples were subjected to SDS-PAGE as before (19) and stained with silver staining. Samples were scanned with a Shimadzu CS-630 dual wavelength densitometer.

RESULTS AND DISCUSSION

In the course of studies on the mechanism of cell vacuolization induced by VacA, we experienced large variations in the potency of different batches of VacA. These variations were associated with different handling of samples with respect to storage. In particular, we noticed that freezing in liquid nitrogen and thawing activated VacA, particularly when phosphate ions were present. Since neutral phosphate buffers acidify upon freezing (21), we tested the effect of exposure of non-frozen VacA to acidic pH values.

Fig. 1 shows that HeLa cells incubated with VacA, exposed for 15 min to pH 2.0, and then neutralized to pH 7.4, develop very large vacuoles, whereas cells exposed to a non-acid-treated VacA are very weakly vacuolized. A quantitative assay of the extent of low pH activation is shown in Fig. 1*A*.

The pH dependence profile of Fig. 2*A* indicates that activation of VacA takes place already at pH 5.5. It is noteworthy that VacA is activated rather than damaged by exposure to a pH as low as 1.5, similar to that of the stomach lumen (22). A sample of VacA in PBS frozen in liquid nitrogen and thawed was activated by this procedure to about one-fourth of the value attained by a corresponding sample exposed to pH 2.0. Activation by slower freezing at −70 and −20 °C is very weak (not shown).

Low pH-induced activation of VacA is very rapid, being essentially complete within 10 s at pH 2.0 at 37 °C (Fig. 2*B*). Once activated, VacA retains its activity at neutral pH for long periods; after 24 h, VacA still has 70% of its original vacuolating activity and, after 3 days, the remaining activity is 20% of the control kept at neutral pH and then low pH-activated before cell assay. This slow rate of VacA inactivation at neutral pH is not due to a decay to the original preactivation state, because VacA cannot any longer be activated by re-exposure to low pH. Control SDS-PAGE gels show no VacA degradation.

The alteration of membrane trafficking at the late endosome level caused by VacA is consistent with an activity displayed in the cytosol. Several bacterial protein toxins with intracellular targets penetrate cells via internalization into acidic intracellular compartments, wherefrom the active toxin domain is released in the cytosol (23). Low pH-activated VacA does not require intracellular acidification because it is fully active in the cell vacuolation assay of Fig. 1, which requires the presence of NH_4^+ ions (10, 11, 18), an agent known to neutralize intracellular acidic compartments. To test the possibility that VacA is activated inside acidic cell compartments, VacA not exposed to low pH was added to HeLa cells in the absence of NH_4^+ ions, for different time periods up to 2 h. Cells were then allowed to vacuolize in the presence of NH_4^+ ions, but no vacuoles were observed (not shown). This result indicates that VacA cannot be activated inside acidic cellular compartments, though control immunofluorescence experiments indicated that VacA had been internalized (not shown).

Low pH activation of VacA is accompanied by a structural change that can be monitored spectroscopically (Fig. 3). The far-UV CD spectrum of VacA at pH 7.4 shows a minimum at 215 nm and has a substantial amount of β-like secondary structure (39%) and a lower content of α-helix (15%) and turn (15%) conformation. A substantial decrease of the CD signal is observed upon acidification to pH 2.0, and this accounts for an estimated increase of β-structure (47%) and a concomitant decrease of turn (9%) with no change in α-helix content. Reneutralization of low pH-exposed VacA causes a further decrease in ellipticity at 215 nm. Variations of secondary structure content of VacA are reasonably consistent with a turn → β-structure transition. However, the far-UV CD spectrum of a protein can be influenced by several factors such as the contribution of aromatic residues (24, 25), a change of the twisting angle of the β-sheets strands (26), as well as a possible change in quaternary structure. However, VacA is frequently found as a heptamer in electron microscopy, and the amount and shape of the VacA heptamer does not change upon low pH treatment.[2] The lack of reversibility and the decrease of CD signal upon reneutralization indicate that VacA undergoes a transition to a conformational state different from those present at pH 7.4 and 2.0.

The fluorescence spectra of Fig. 3*B* provide another evidence of the VacA structural transition induced by low pH. At pH 7.4, VacA has an emission maximum at 345 nm, indicating that the 10 Trp residues of VacA are, on average, solvent-exposed (26). In addition, the absence of Tyr fluorescence indicates a strong Tyr-Trp energy transfer, and this is a useful structural probe of

VacA structural changes. Upon acidification, VacA shows a reduced fluorescence quantum yield (60–65%) (Fig. 3*B*), with respect to non-acid-treated VacA solutions (100%), whereas the wavelength of maximum fluorescence emission is red-shifted of about 2 nm. Fluorescence measurements conducted on model compounds (N^{α}-acetyl-L-tyrosinamide:N^{α}-acetyl-L-tryptophanamide in a molar ratio of 2.6:1, the one of VacA) demonstrate that acid quenching of Trp fluorescence accounts for about 90% of the Trp quantum yield decrease observed on acidification to pH 2.0. The remaining decrease may result from change of solvent exposure of Trp residues and/or protonation of neighbor groups. After reneutralization, in agreement with the CD findings, the Trp quantum yield is further reduced with respect to that observed at pH 2.0. The high degree of Tyr-Trp energy transfer at pH 2.0 and after reneutralization indicates that VacA is still highly structured. Only denaturation induces a distinct band of Tyr fluorescence at 303–305 nm and a shift of Trp emission to 352 nm (Fig. 3*B*).

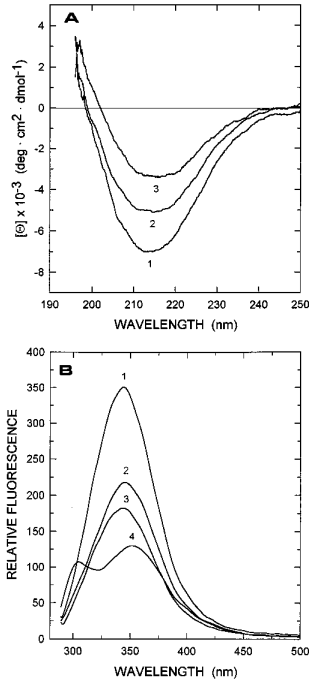

FIG. 3. **Circular dichroism (*A*) and emission fluorescence (*B*) spectra of VacA excited at different pH values.** Spectra of VacA (90 μg/ml for CD and 32 μg/ml for fluorescence) were taken at 37 °C in PBS at pH 7.4 (*curve 1*), in PBS at pH 2.0 (*curve 2*), and after reneutralization (*curve 3*) as detailed under "Materials and Methods." *Curve 4* of *panel B* refers to VacA treated with 3.5 M guanidinium chloride.

Different structural states of proteins can be monitored with high sensitivity by determining their susceptibility to proteinases (19, 27). Fig. 4 shows that VacA is fragmented by Pronase and proteinase K differently, depending on a preliminary exposure to pH 2.0. Identification of the sites of proteolytic cleavage is under way and can provide relevant information on exposed loops (27). The present data clearly indicate that the two VacA forms are structurally different and are therefore degraded differently. Fig. 4*B* shows that VacA at pH 2.0 is very resistant to pepsin, the major proteinase of stomach juice, whereas BSA is completely degraded.

Spectroscopic and limited proteolysis data indicate that VacA can exist in at least three different structural states: neutral VacA (cytotoxin never exposed to low pH), acid VacA (VacA at low pH), and reneutralized VacA (VacA exposed to low pH and returned to neutrality). Reneutralized VacA is very active in inducing cell vacuolization, whereas VacA kept at neutral pH is virtually inactive. Some reneutralized form of VacA was inadvertently obtained previously by freezing in phosphate-containing buffers (10, 11, 13). The activity of acid VacA cannot be measured because of the very nature of the cell vacuolization assay, which requires cultivation of epithelial cell for several hours. For a similar reason, also limited proteolysis only monitors the neutral and reneutralized states of VacA.

The present findings are very relevant to the characterization of the structure-function relationship of VacA, a major virulence factor in the pathogenesis of gastrointestinal ulcers (9–16). At the same time, they may contribute to the understanding of the pathogenesis of duodenal ulcers. In fact, it is known that *H. pylori* penetrates the mucus layer of the stomach and adheres to the apical portion of parietal epithelial cells (5, 6), whereas its presence is not reported on the duodenum epithelium. Stomach justamucosal pH is kept between 6 and 7 by the mucus gel that traps bicarbonate ions and prevents back diffusion of HCl from the stomach lumen (28–30). In *H. pylori*-infected patients, justamucosal pH of the gastric body is lowered to the mean value of 5.7 (31). Here we show that VacA is strongly activated at pH <6.0 down to pH 1.5, without being affected by such an extreme pH. At the same time, VacA is strongly resistant to pepsin at pH 2.0. Hence, it can be envisaged that some VacA molecules are released and activated in the stomach juice and pass through the pylorus in the intestine. Here, activated VacA can induce vacuolization of epithe-

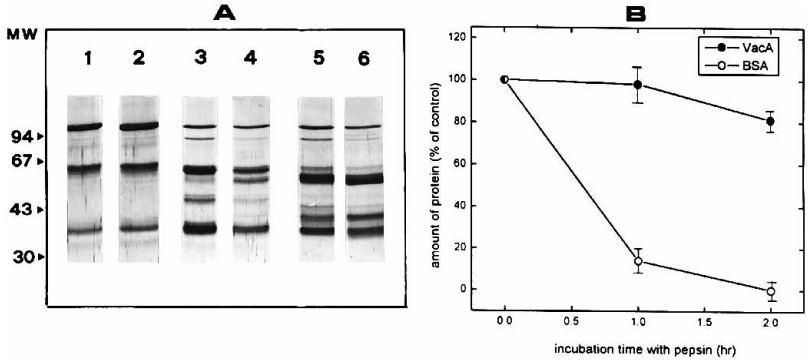

FIG. 4. **Limited proteolysis fragmentation patterns of VacA with Pronase and proteinase K.** *A*, *odd*- and *even*-numbered *lanes* refer to non-acid-treated and acid-treated VacA samples, respectively. *Lanes 1* and *2*, VacA not treated with proteases; *lanes 3* and *4*, VacA incubated with Pronase; *lanes 5* and *6*, VacA incubated with proteinase K. All proteolytic digestions were carried out in PBS at pH 7.8 for 2 h at 37 °C. 4.8 μg of VacA were loaded in each SDS-PAGE *lane*, and samples were stained with silver staining. *B*, VacA (●) and BSA (○) were incubated at pH 2.0 for 15 min at 37 °C and then for an additional 1 or 2 h with pepsin. 3.3 μg of VacA or BSA were loaded in each SDS-PAGE lane and, after silver staining, the region of the gel containing the uncleaved protein band was scanned with a densitometer. Data are percentages of the amount of protein of control samples incubated under the same conditions without pepsin, taken as 100%. The average of results obtained on two different batches of VacA is shown, and *bars* represent the range of values obtained.

lial cells of the duodenum, before being digested by intestinal proteases, which would thus protect later portions of the intestine. Such a process could contribute to the development of duodenal lesions without the physical presence of *H. pylori*.

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