Activation of Helicobacter pylori VacA Toxin by Alkaline or Acid Conditions Increases Its Binding to a 250-kDa Receptor Protein-tyrosine Phosphatase β*

Kinnosuke Yahiro‡‡, Takuro Niidome‡‡, Miyuki Kimura‡‡, Tomomitsu Hatakeyama‡‡, Haruhiko Aoyagi‡‡, Hisao Kurazono‡‡, Ken-ichi Imagawa‡‡, Akiko Wada‡‡, Joel Moss‡‡*, and Toshiya Hirayama‡‡‡‡

From the ‡Department of Bacteriology, Institute of Tropical Medicine, and ‡Department of Applied Chemistry, Faculty of Engineering, Nagasaki University, Nagasaki 852-8523, Japan, the §Department of Microbiology, Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba-shi 305-0006 Japan, †Orsuka Pharmaceutical Co., Ltd, Tokushima 771-0192, Japan, and the **Pulmonary-Critical Care Medicine Branch, NHLBI, National Institutes of Health, Bethesda, Maryland 20892

Helicobacter pylori, a Gram-negative gastric bacterium, secretes VacA, a cytotoxin that causes vacuolar degeneration of susceptible cells. Velocity sedimentation analysis showed that treatment of VacA at alkaline pH led to disassembly of VacA oligomers, an observation reported previously for acid-treated VacA. Exposure of VacA to acid or alkali increased its binding to AZ-521 cells, as shown by indirect immunofluorescence and flow cytometry. Moreover, immunoprecipitates with polyclonal antibodies against VacA from AZ-521 cells previously exposed to acid- or alkali-treated VacA had a 250-kDa glycoprotein containing galactose-β(1–3)-N-acetylgalacosamine and galactose-β(1–4)-N-acetylgalactosamine. p250, purified by chromatography on peanut agglutinin affinity and Superose 6 columns, contained N-terminal and internal amino acid sequences of YRQQRKLVEEIGWSYT and LIQHDHILEATQDDY, respectively. These sequences are identical to those of a receptor protein-tyrosine phosphatase (RPTPβ)/PTPζ; in agreement, p250 reacted with anti-human RPTPβ monoclonal antibody. Immunoprecipitation with anti-human RPTPβ antibody of solubilized membrane preparations previously incubated with VacA or heat-inactivated VacA demonstrated that RPTPβ bound native, but not denatured, VacA. Acidic and alkaline treatments were associated with activation of VacA and increased binding to the cell surface RPTPβ.

Helicobacter pylori causes chronic, active gastritis and peptic ulcer disease and is a risk factor for gastric cancer (1). Although most H. pylori infections are asymptomatic, H. pylori type I secretes a potent vacuolating cytotoxin (VacA) and is associated with severe symptomatic gastritis. VacA causes gastric mucosal damage in mice (2, 3) and has been proven epidemiologically to be a virulence factor associated with peptic ulcer (4, 5). VacA, a 87-kDa mature toxin, was purified from the culture supernatant of H. pylori toxin-positive strain 60190 (ATCC49503) (6); a 95-kDa VacA was also isolated from cytotoxic strain CCUG 17874 (7). The VacA gene has a 3864-base pair open reading frame, encoding a precursor protein for the mature 87-kDa VacA (5), and a 4200-base pair open reading frame for the precursor of the 95-kDa VacA (3). Incubation of VacA at pH 2.0 increased its vacuolating activity and its resistance to acid and pepsin degradation (8). It was notable that acid-activated VacA contributed to the development of duodenal lesions, independent of H. pylori (8).

H. pylori grows persistently in the mucus layer of gastric tissue by means of a potent urease which produces ammonia; this product plays a significant role in the neutralization of the acidic environment of the stomach (9) and allows the bacteria in vivo to adhere to the apical plasma membrane of surface epithelial cells in the pyloric antrum (10–12). Ultrastructural analysis of gastric mucosa infected with H. pylori showed that bacteria adhere to gastric epithelial cells on small cellular projections referred to as adherence pedestals (10). In vitro, H. pylori adhered to AGS human gastric adenocarcinoma cells; H. pylori appeared to be partially enclosed by plasma membrane, which was associated with a loosely organized network of actin filaments (13). It is probable that a non-acidic or alkaline environment is created by ammonia produced from urea secreted by gastric epithelial cells by action of H. pylori urease, even though the gastric juxtamucosal pH of patients infected with H. pylori is 5.7 (14). We, therefore, examined VacA activation in buffers of different pH and found that it was activated not only under acidic, but also alkaline, conditions. Rapid vacuolation of AZ-521 cells incubated with acid- or alkali-treated VacA was associated with its enhanced binding to a 250-kDa surface glycoprotein, termed p250. Amino acid sequence data and immunological analysis showed that p250 is receptor protein-tyrosine phosphatase β (RPTPβ).¹

MATERIALS AND METHODS

Preparation of Purified VacA—The toxin-producing strain H. pylori ATCC49503 was used as the source of VacA for purification according to our published procedure (15). In brief, after growth of H. pylori in Brucella broth containing 0.1% β-cyclodextran at 37 °C for 3–4 days with vigorous shaking in a controlled micro-aerophilic atmosphere of 10% O₂ and 10% CO₂, VacA was precipitated from culture supernatant

¹ The abbreviations used are: RPTPβ, receptor protein-tyrosine phosphatase β; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PVDF, polyvinylidene difluoride; NBU, neutral red uptake; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; EGF, epidermal growth factor; PNA, peanut agglutinin; DSA, D. stramonium agglutinin.
with 50% saturated ammonium sulfate and purified by column chromatography on hydroxyapatite, Superose 6HR 10/30, and Resource Q, followed by filtration (0.45-μm filter). Purified VacA (200 μg/ml) was stored in TBS buffer (60 mM Tris-HCl buffer, pH 7.7, containing 0.1 mM NaCl).

**Assay of Vacularizing Activity**—Human gastric adenocarcinoma cell line AZ-521 (Culture Collection of Health Science Research Resources Bank, Japan Health Science Foundation) was seeded in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in 96-well culture plates (2 × 10^4 cells in 80 μl/well) and cultured in monolayers for 24 h in a 5% CO_2 atmosphere at 37 °C. Samples of 20 μl of VacA were added to achieve the indicated final concentrations and cells were incubated for an additional 8 h at 37 °C. To quantify vacuolating activity, uptake of neutral red into the vacuoles in VacA-treated cells was determined. Cells were incubated for 5 min at room temperature with 50 μl of freshly prepared 0.05% neutral red in PBS containing 0.3% BSA, and then washed three times with 0.1 ml of PBS containing 0.3% BSA. After addition of 0.1 ml of 70% ethanol in water containing 0.4% HCl, absorbance at 540 nm (A_540) was measured. Vacuolating activity was determined by subtracting the A_540 of cells incubated without VacA from the A_540 of VacA-treated cells.

To evaluate the effect of acid or alkali treatment on vacuolating activity, VacA was adjusted to indicated pH by the addition of 1 N HCl or 1 N NaOH, incubated at 30 °C for 10 min and then neutralized to pH 7.7 with 1 N NaOH or 1 N HCl, respectively. The resulting samples were harvested in TNE buffer, containing 40 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 mM EDTA, washed twice in PBS, and suspended in 1 ml of the same solution, and incubated at 4 °C for 30 min with 10 μl of 100 mM NaCl containing either 60 mM Tris, pH 7.7, 100 mM glycine/HCl, pH 3.0, or 100 mM glycine/NaOH, pH 10 and centrifuged at 10 °C for 7 h at 40,000 rpm in an SW50.1 rotor (Beckman Instruments). Fractions (550 μl) from the tops of the gradients were analyzed by 7.5% SDS-PAGE and immunoblotting with anti-VacA antibody. After aliquots (400 μl) of each fraction were dialyzed against TBS buffer and concentrated to 100 μl using Centricon concentrator with Amicon Centriflip-100, 10 μl of each sample was assayed for vacuolating activity. Standard marker proteins, i.e., BSA (4.6 S), catalasize (11.2 S), and thyroglobulin (19 S), were centrifuged in parallel gradients.

**Glycoler Density Gradient Centrifugation**—Samples (100 μl) of the untreated VacA (40 μg) or VacA that had been incubated for 10 min at 30 °C at pH 1.5, 3, 10, or 11.5 with or without subsequent neutralization to pH 7.7 were layered on 5-ml 10–35% glycerol gradients prepared in 100 mM NaCl containing either 60 mM Tris, pH 7.7, 100 mM glycine/HCl, pH 3.0, or 100 mM glycine/NaOH, pH 10 and centrifuged at 10 °C for 7 h at 48,000 rpm in an SW50.1 rotor (Beckman Instruments). Fractions (550 μl) from the tops of the gradients were analyzed by 7.5% SDS-PAGE and immunoblotting with anti-VacA antibody. After aliquots (400 μl) of each fraction were dialyzed against TBS buffer and concentrated to 100 μl using Centricon concentrator with Amicon Centriflip-100, 10 μl of each sample was assayed for vacuolating activity. Standard marker proteins, i.e., BSA (4.6 S), catalasize (11.2 S), and thyroglobulin (19 S), were centrifuged in parallel gradients.

**Flow Cytometric Analysis of Toxin Binding**—AZ-521 cells (1 × 10^4), were harvested in TNE buffer, containing 40 μl Tris-HCl, pH 7.5, 150 mM NaCl, and 1 μl EDTA, washed twice in PBS, and suspended in 1 ml of TNE buffer containing 2% BSA. Samples (20 μl) were incubated at 4 °C for 30 min with 10 μl of 0.62–40 μg of untreated VacA, VacA exposed to pH 1.5 or 11.5 followed by neutralization, or to heat-inactivated VacA (95 °C, 10 min). After two washes with 500 μl of PBS containing 2% BSA, anti-VacA antibody was added, followed by incubation at 4 °C for 30 min. Cells were washed twice with PBS containing 2% BSA, suspended in 1 ml of the same solution, and incubated at 4 °C for 30 min with 5 μg of fluorescein isothiocyanate (FITC)- labeled goat antimouse IgG (H+L) (Amersham Pharmacia Biotech). After two washes with PBS containing 2% BSA, samples of 10,000 cells were analyzed by flow cytometry (Becton Dickinson Immunocytometry System) with excitation at 488 nm and emission at 530 nm.

**Immunoprecipitations**—Cultured cells (5 × 10^7) were harvested in 1 ml of TNE buffer and washed twice with PBS. Protein of the cell surface was biotinylated at 4 °C for 30 min according to the manufacturer's specifications (Amersham Pharmacia Biotech; ECL protein biotinylation module, no. RPN 2202). Biotinylated cells were lysed with 0.5 ml of Sol buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM glycerol, 5 μg of leupeptin, 10 mM iodoacetate, and 1% Triton X-100) for 15 min on ice. After centrifugation (20 min, 10,000 × g), the supernatant was filtered. The filtrate was diluted with an equal volume of chilled water and applied to a column of PNA agarose (1 ml bed volume), which was washed with DW buffer (25 mM Tris, 0.3% NaCl, 5% glycerol, 0.3% Triton X-100, and leupeptin, 5 μg/ml, pH 7.5, and eluted with DW buffer containing 0.3% T-digalactose. Fractions, in which p250 was detected by immunoprecipitation using VacA and anti-VacA antibody, were pooled and concentrated by SpeedVac. The concentrated sample was transferred to a column of Superox 6 (1 × 30 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 0.15% NaCl, 2.5% glycerol, 0.5% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, and 0.5 μg/ml leupeptin.

**Peptide Sequence Analysis**—After SDS-PAGE in 5% gels, proteins were transferred to a PVDF membrane (Problot, Applied Biosystems), stained with Coomassie Brilliant Blue, reduced, and carboxymethylated (23), and digested with Asp-nexin protease I (Wako Pure Chemical Industries, Osaka, Japan). Peptides released from the membrane were fractionated by narrow-bore liquid chromatography on a Smart system (Amersham Pharmacia Biotech) using a nRPC C2/C18 SC2.1/120 column (Amersham Pharmacia Biotech), and sequenced (model 477A/120A sequencer; Applied Biosystems Division, Perkin Elmer Corp.).

**Other Methods and Chemicals**—Protein was measured by the method of Bradford using bovine serum albumin as standard (24). Molecular mass standards for SDS-PAGE were phosphorylase b (97,4 kDa), BSA (66 kDa), hen egg albumin (46 kDa), and carbonic anhydrase (31 kDa). High molecular mass markers for glycerol density gradient centrifugation, BSA (4.6 S), catalase (11.2 S), and thyroglobulin (19 S), were obtained from Amersham Pharmacia Biotech. N-Glycosidase F and O-glycosidase were purchased from Roche Molecular Biochemicals. β-Cyclodextran was kindly supplied by Teijin (Tokyo, Japan). Other reagents were of analytical grade.
RESULTS

Stimulation of Vacuolating Activity by Acidic and Alkaline Conditions—Fig. 1 shows the time course of vacuolation of cultured cells with VacA (final concentration, 30 nM (panel A), 60 nM (panel B), 120 nM (panel C), and 240 nM (panel D)) that had been treated at 30 °C for 10 min at pH 1.5 or 11.5 followed by neutralization to pH 7.7. Cells exposed to the treated VacA were vacuolated at similar rates and much more rapidly than cells incubated with untreated VacA. Time-dependent vacuolating activity, as measured by NRU, was increased about 3-fold by incubation for 8 h with 120 nM acid- or alkali-treated VacA. Significant activation was observed at pH values lower than 5.5 and greater than 9.5 (Fig. 2). As shown in Fig. 3, VacA (3.75–240 nM) treated at pH 1.5 or 11.5 showed greater vacuolating activity than untreated VacA. Moreover, specific activity of VacA (up to 60 nM) treated at pH 3 or 10 was significantly lower than that of VacA treated at pH 1.5 or 11.5.

Effect of pH on VacA Aggregation—Velocity sedimentation analysis of acid-treated VacA had demonstrated acid-induced dissociation of the oligomeric form and its reassembly on neutralization (25). To determine whether alkaline treatment of VacA similarly induced disassembly of VacA oligomers, we used velocity sedimentation in glycerol density gradients followed by Western blotting of fraction samples with anti-VacA antibody (Fig. 4A). After incubation at pH 1.5 or 11.5 for 10 min and centrifugation at the same pH, VacA was essentially entirely found in fractions containing BSA; after incubation and sedimentation at pH 7.7, all VacA was in fractions corresponding to proteins of 19–22S (Fig. 4). VacA incubated and centrifuged at intermediate pH (pH 3 or 10) was divided between the two gradient regions. Regardless of treatment pH, however, after sedimentation at pH 7.7, all VacA was found in fraction 6; this VacA sedimented more rapidly than thyroglobulin (19 S) and corresponded to ~22 S (25). Thus, the extreme in alkaline, as well as acidic pH, resulted in disassembly of native VacA oligomers. These results indicate that VacA dissociated by treatment at pH 11.5, like acid-treated VacA, reassociated on neutralization and sedimented at ~22 S.

Vaculating activity of each fraction was determined after dialysis against TBS buffer. After taking into account the yield of standard marker protein, total recovery of vacuolating activity of all VacA exposed to pH 11.5 or 1.5 was about 226–289% of untreated VacA (Fig. 4B). The vacuolating activity of fractions 2 and 3 containing VacA treated at pH 11.5, as well as at pH 1.5 (Fig. 4B, b and c), was remarkably increased. After incubation at pH 1.5 (Fig. 4B, d) or 11.5 (Fig. 4B, e) and sedimentation at pH 7.7, VacA in fraction 6 showed higher vacuolating activity than did untreated VacA (Fig. 4B, a). These results suggest that VacA may be activated by exposure to alkaline conditions and reassociated after neutralization. The findings are similar to those observed with acid-treated VacA.

Binding of Acid- and Alkaline-treated VacA to AZ-521 Cells—Cells exposed to VacA that had been incubated at alkaline or acidic pH followed by neutralization were much more rapidly vacuolated than those incubated with the untreated VacA. We, therefore, evaluated using fluorescence-activated cell sorting, the binding to AZ-521 cells of VacA after treatment at pH 1.5 or 11.5 (Fig. 5). Mean fluorescence intensity of cells incubated with VacA previously exposed to pH 1.5 or 11.5 was 145 and 83, respectively, versus 40.7 for cells incubated with 120 nM untreated VacA. Thus, fluorescence intensity, which is an indirect measure of VacA bound to AZ-521 cells, was significantly higher (about 1.3–3 fold) with treated than with untreated VacA concentrations from 3.75 to 240 nM (Fig. 6). These results suggest that treatment of VacA at pH 1.5 or 11.5 increased its binding to AZ-521 cells. Two proteins of 140 and 250

Fig. 1. Time course of VacA-induced vacuolization of AZ-521 cells. Purified VacA was incubated at 30 °C for 10 min at pH 1.5 or 11.5 followed by neutralization to pH 7.7. Cells in a 96-well microplate were incubated with 2.5–20 μg of treated or untreated VacA (final concentration, 30 nM (panel A), 60 nM (panel B), 120 nM (panel C), and 240 nM (panel D)) for the indicated time before measurement of neutral red uptake (A<sub>405</sub>) as an index of vacuolation. ○, VacA treated at pH 1.5; △, VacA treated at pH 11.5; □, untreated VacA. Data are means of three independent assays run in duplicate ± S.D. Optical densities of cells incubated for 8 h without VacA were in the range of 0.02–0.03. The experiment was repeated three times with similar results.

Fig. 2. Effect of pH on vacuolating activity of VacA. Purified VacA (120 nM) in TBS buffer was exposed to the indicated pH for 10 min at 30 °C before neutralization to pH 7.7. AZ-521 cells were incubated with the treated VacA for 8 h. The extent of vacuolation was assayed again by NRU. ○, VacA; □, TBS buffer. Data are means of three independent assays run in duplicate ± S.D. The experiment was repeated three times with similar results.

Fig. 3. Effect of pH on vacuolating activity of VacA. AZ-521 cells in a 96-well microplate were incubated with indicated concentrations of treated or untreated VacA for 8 h before measurement of NRU. ○, untreated VacA; ●, VacA treated at pH 1.5; △, VacA treated at pH 3; ■, VacA treated at pH 10; □, VacA treated at pH 11.5. The experiment was repeated three times with similar results.
kDa on the cell surface of AZ-521 were immunoprecipitated with anti-VacA antibody and protein A-Sepharose CL-4B from lysates of cells that had been incubated with active, but not with heat-inactivated, VacA (Fig. 7). The dense bands of p250

FIG. 4. The effect of acid and alkaline treatments on velocity sedimentation of VacA in glycerol density gradients. Before application to the gradient, VacA was treated for 10 min at 30 °C as indicated. After centrifugation, fractions were collected from the top of each gradient (5 ml), and samples of each fraction (500 μl) were subjected to SDS-PAGE in 7.5% gels. VacA was detected by Western blotting with anti-VacA antibody (A). Only regions of the gels containing the 87-kDa protein are shown. The positions of standards sedimented in a parallel gradient are shown at the bottom for BSA (4.6 S), catalase (11.2 S), and thyroglobulin (19 S). For assay of VacA vacuolating activity in each fraction after incubation at pH 1.5 or 11.5 with or without neutralization (B), AZ-521 cells in a 96-well microplate were incubated with sample for 8 h before measurement of NRU as described in the text. The experiment was repeated three times with similar results.

FIG. 5. Binding to AZ521 cells of VacA treated at pH 1.5 or 11.5 followed by neutralization. Cells were incubated with heat-inactivated VacA (first peak in each panel) or with untreated VacA (A) or with VacA neutralized after exposure to pH 1.5 (B) or pH 11.5 (C), then for 30 min with anti-VacA antibody, and finally for 30 min with Fluoro LinkTM-Cy3-labeled goat anti-rabbit IgG (H+L), followed by FAScan analysis. The results are plotted as relative cell number versus Fluoro LinkTM-Cy3 fluorescence and are representative of three experiments; 10,000 cells were analyzed per sample. Mean fluorescence intensity of cells is recorded above each peak. The experiment was repeated three times with similar results.

FIG. 6. Effect of treatment at pH 1.5 or 11.5 on VacA binding to AZ521 cells. Cells were incubated for 30 min at 4 °C with indicated concentrations of untreated VacA (■) or VacA that had been exposed to pH 1.5 (●) or pH 11.5 (▲) and neutralized, followed by a 30-min incubation with anti-VacA antibody and additional 30 min with Fluoro LinkTM-Cy3-labeled goat anti-rabbit IgG (H+L), followed by flow cytometry, as described in the legend of Fig. 5 and under “Materials and Methods.” The experiment was repeated three times with similar results.
followed by neutralization (Fig. 7A). Densitometric scanning of the bands detected by immunoprecipitation clearly indicates 5.1- or 3.7-fold stimulation of VacA binding to p250 after treatment, respectively, at pH 1.5 or 11.5. After incubation with anti-VacA antibody, antibody-bound proteins were collected by addition of protein A-Sepharose CL-4B. Immunoprecipitated proteins, separated by SDS-PAGE in 5% gels, were transferred to PVDF membranes, which were incubated with horseradish peroxidase-conjugated streptavidin, followed by incubation with anti-digoxigenin Fab fragments conjugated to alkaline phosphatase, were visualized after reaction with 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate. Panel C shows p250 after incubation with HRP-conjugated streptavidin followed by ECL detection. Positions of molecular mass standards (kDa) are on the left; position of p250 is on the right. The experiment was repeated three times with similar results.

**Fig. 7. Effect of treatment of VacA at acid or alkaline pH on binding to cell surface proteins.** A, electrophoretic patterns of the proteins immunoprecipitated from AZ-521 cells. Membrane proteins were biotinylated at 4 °C for 30 min. The cells were then lysed with Sol buffer, the lysate was centrifuged for 20 min at 100,000 × g, and the supernatant was incubated with 3.0 μg of heat-inactivated VacA (lane 1), untreated VacA (lane 2), or VacA neutralized after treatment at pH 1.5 (lane 3) or pH 11.5 (lane 4). After incubation with anti-VacA antibody, antibody-bound proteins were collected by addition of protein A-Sepharose CL-4B. Immunoprecipitated proteins, separated by SDS-PAGE in 5% gels, were transferred to PVDF membranes, which were incubated with horseradish peroxidase-conjugated streptavidin, followed by ECL detection. Positions of molecular mass standards (kDa) are on the left and right. Arrows show the locations of p250 and p140. B, densitometric scans of immunoprecipitated proteins visualized by ECL. Arrows 1 and 2 show the locations of p250 and p140, respectively. The experiment was repeated three times with similar results.

Proteins immunoprecipitated from biotinylated cells that had been incubated with heat-inactivated VacA (lane 1) or native VacA (lane 2) were separated by SDS-PAGE in 5% gels and transferred to PVDF membranes. PNA- and DSA-binding proteins (panels A and B, respectively), detected by reaction with lectin conjugated to digoxigenin followed by incubation with anti-digoxigenin Fab fragments conjugated to alkaline phosphatase, were visualized after reaction with 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate. Panel C shows p250 after incubation with HRP-conjugated streptavidin followed by ECL detection. Positions of molecular mass standards (kDa) are on the left; position of p250 is on the right. The experiment was repeated three times with similar results.

Differences, however, among the intensities of the p140 bands observed in three lanes. These results indicate that the treatment of VacA at pH 1.5 or 11.5 followed by neutralization contributed to the increased binding of the treated VacA to p250.

**Structural Characteristics of p250—** To identify carbohydrate moieties, lectin binding to p250 was quantified. PNA and DSA reacted strongly with p250, indicating the presence of galactose-β(1–3)-N-acetylgalactosamine, and galactose-β(1–4)-N-acetylglucosamine, respectively (Fig. 8). After incubation with N-glycosidase F or O-glycosidase, the biotinylated surface proteins of AZ-521 cells were immunoprecipitated using native VacA and heat-inactivated VacA (Fig. 9). The samples immunoprecipitated using native VacA before (lane 2) and after (lane 4) N-glycosidase F treatment contained 250- and 240-kDa proteins, respectively. These results show that p250 has N-linked glycan and that the N-linked glycan of p250 is required for the binding to VacA. A significant molecular change in p250 was not observed after treatment with O-glycosidase (lane 6).

Based on the characteristics of sugar moieties of p250, which bind to PNA, we purified p250 from AZ-521 cells by PNA and Superose 6 chromatography. The major protein in the effluent from the PNA column was p250; some p140 was also detected. Proteins of 140 and 250 kDa from the surface of AZ-521 cells were immunoprecipitated with anti-VacA antibody and protein A-Sepharose CL-4B after incubation of the effluent with VacA, but not with inactivated VacA, suggesting that both p140 and p250 could bind VacA (data not shown). After Superose 6 chromatography, p250 in the effluent appeared as a single band on silver-stained 5% SDS-polycrylamide gels (Fig. 10A). The N-terminal amino acid sequence of purified p250 was YRQQKRLVEEIGWSYT. An internal sequence obtained from a peptide isolated after *Achromobacter protease* I hydrolysis was LIQDHELEATQDDY. These amino acid sequences are consistent with the position 2–15 or 7–22 sequence and the position 2140–2154 or 2145–2159 sequence of RPTPβ reported by Krueger and Saito (26) or Levy et al. (27), respectively, suggesting that p250 is RPTPβ. On immunoblotting (Fig. 10B), p250 was a single band that reacted with anti-human RPTPβ monoclonal antibody, which had been prepared by immunizing mice with a polypeptide representing amino acids 2098–2307 of human RPTPβ. Thus, these data add strong support to the hypothesis that p250 is the full-length form of RPTPβ.

To examine the specific binding of p250 to VacA, co-immunoprecipitation was carried out using anti-human RPTPβ monoclonal antibody, which had been prepared by immunizing mice with a polypeptide representing amino acids 2098–2307 of human RPTPβ.
Samples were then incubated with heat-inactivated VacA (lanes 1 and 2) or with N-glycosidase F (lanes 3 and 4) or O-glycosidase (lanes 5 and 6) at 37 °C for 3 h. Samples were then incubated with heat-inactivated VacA (lanes 1, 3, and 5) or native VacA (lanes 2, 4, and 6) before immunoprecipitation and separation of precipitated proteins by SDS-PAGE in 5% gels following by transfer to PVDF membranes; p250 was visualized by incubation with HRP-conjugated streptavidin followed by ECL detection. Positions of molecular mass standards are on the left, and calculated values for reactive bands are on the right. The experiment was repeated three times with similar results.

FIG. 9. Immunoprecipitation of p250 after treatment with N-glycosidase F and O-glycosidase. Proteins solubilized from biotinylated cells were incubated without (lanes 1 and 2) or with N-glycosidase F (lanes 3 and 4) or O-glycosidase (lanes 5 and 6) at 37 °C for 3 h. Samples were then incubated with heat-inactivated VacA (lane 1 in each panel) or VacA (lane 2 in each panel) at 4 °C for 1 h, samples were incubated with anti-human RPTPβ monoclonal antibody at 4 °C overnight. Following addition of protein A-Sepharose CL-4B, and incubation for 1 h, immunoprecipitated proteins were separated by SDS-PAGE in 5% gel and transferred to PVDF membranes. Proteins were detected using antibodies against VacA (panel A) or RPTPβ (panel B).

FIG. 10. SDS-PAGE of p250 purified from AZ-521 cell. p250 (~20 ng), purified from AZ-521 cells by chromatography on PNA affinity matrix and Superose 6, was subjected to SDS-PAGE in 5% gels and visualized after silver staining (panel A) or immunoblotting using anti-RPTPβ monoclonal antibody (panel B).

VacA or heat-inactivated VacA was incubated with p250 before treatment with anti-human RPTPβ monoclonal antibody and protein A-Sepharose CL-4B. 87-kDa VacA was visualized by Western blotting with anti-VacA antibody in the immunoprecipitate from the sample containing native VacA (lane 2), but not heat-inactivated VacA (lane 1). p250 was present in each sample (Fig. 11B). Thus, p250 serves as a surface receptor for VacA and can bind specifically to native but not heat-inactivated VacA.

DISCUSSION

VacA has been implicated in the etiology of peptic ulcer, although its role in pathogenesis of the disease and the mechanism by which VacA exerts its effects are both unclear. The fact that VacA is activated by exposure to low pH (8) is intriguing when we consider the function of VacA in H. pylori gastric infection. In this study, we showed that VacA exposed to alkaline or acid conditions, with subsequent neutralization, exhibits enhanced vacuolating activity; the acid or alkali-activated VacA appears to bind a cell surface receptor protein of ~250 kDa. N-terminal and internal amino acid sequence is consistent with the hypothesis that p250 is RPTPβ.

de Bernard et al. (8) previously reported that acid activation of VacA altered its circular dichroism, fluorescence spectrum, and proteolytic pattern. The fluorescence emission spectrum of the native VacA differed from those of acid- and alkali-activated VacA (data not shown). The fluorescence spectra of the activated neutralized proteins were similar, with a 4-nm blue shift relative to native VacA, due to broadening of the peak. These data suggest that alkali-activated VacA undergoes a conformational change after neutralization similar to that of acid-activated VacA. In agreement, acid- and alkali-activated VacA display quite similar proteolytic cleavage patterns (data not shown). These results suggest that drastic pH changes followed by neutralization induce tertiary structural changes in VacA, which promote its interaction with a surface receptor, p250, and are not reversed by neutralization.

More recently, it was shown by deep-etch electron microscopy that the intact VacA oligomer consists of 12 subunits assembled into two interlocked six-membered arrays. Disassembly of VacA oligomers occurs concomitantly with the acid-induced activation and cytotoxicity (25). Neutralization of VacA after exposure to acidic pH resulted in seven-sided VacA oligomers, which are different from the six-sided native VacA oligomers (25). By analysis of VacA association with model lipid membranes using atomic force microscopy, Czajkowsky et al. (28) proposed that the native VacA oligomer consists of 12 subunits, which disassemble at low pH and reassemble into membrane-spanning hexamers, consistent with the proposal that low pH-triggered pore formation is likely a critical step in VacA activity. In the sedimentation velocity analysis of untreated and acid- or alkali- treated VacA (Fig. 4), the neutralized VacA as well as the untreated VacA sedimented as molecules of ~22 S. Treatment of VacA at high pH such as pH 11.5 resulted in disassembly of VacA oligomers, as had been reported at acidic pH (25, 28). The extent of disassembly of native VacA oligomers at low and high pH was proportional to VacA specific activity after neutralization (Fig. 3). The tertiary structural changes induced by the alkali treatment are not clearly defined. Further investigation is necessary to determine whether reassembly of alkali-treated VacA following neutralization to pH 7.7 gives rise to tertiary structural changes similar to those in seven-sided VacA oligomers (25) or six-sided VacA oligomers (28).

It is clear that cells exposed to VacA treated at acid or alkaline pH were much more rapidly vacuolated than cells incubated with untreated VacA. Although the mechanism that induces an activated state of the vacuolating toxin has been the subject of much speculation, pore formation by VacA is likely to be related to its toxic effect on target cells (28). Rapid vacuolation of the cells treated with VacA that had been exposed to acid or alkaline conditions was correlated with enhanced binding to the cells via a VacA receptor (Figs. 5–7). With regard to the mode of action of aerolysin, a pore forming hemolysin produced by Aeromonas hydrophila, it is argued that the function of aerolysin protein receptor is to concentrate the toxin on the cell surface, facilitating oligomerization of aerolysin and leading to formation of a channel in the cell membrane. The resulting selective permeabilization to small ions may lead to decreased plasma membrane potential and/or hemolysis. By
using indirect immunofluorescence and flow cytometry binding of VacA to specific high affinity cell surface receptors was shown. Furthermore, this interaction was shown to be necessary for cell intoxication (29).

p250 glycoprotein served as a receptor for VacA on AZ-521 cells; a second protein, p140, was also commonly detected in VacA-susceptible cells (15). p250 has both galactose-\(1-(1-3)-N\)-acetylgalactosamine and galactose-\(1-(1-4)-N\)-acetylglucosamine in its carbohydrate moieties, consistent with the experimental data showing susceptibility of p250 to \(N\)-glycosidase F digestion (Fig. 9). At present, it appears that removal of \(N\)-glycans from p250 by \(N\)-glycosidase does not alter binding to VacA.

Three isoforms of RPTP\(\beta\) are known: RPTP\(\beta\)-A, the full-length form; RPTP\(\beta\)-B, the short form lacking a serine-glycine-rich region in the extracellular domain; and RPTP\(\beta\)-S, the form lacking transmembrane and intracellular domains (30). Among these isoforms of RPTP\(\beta\), the full-length form RPTP\(\beta\)-A was found by immunoblotting to be associated with p250 (Fig. 10B). Many extracellular agents induce their cellular responses by regulating the tyrosine phosphorylation of target proteins such as the EGF receptor. Indeed, a recent report suggests that VacA interferes with EGF-activated signal transduction pathways, which are known to be essential for cell proliferation and ulcer healing (31). Our studies might provide an additional explanation for these findings. Increased binding of acid- or alkali-activated VacA to the p250 phosphatase may alter its activity and possibly accelerate dephosphorylation of tyrosine on proteins such as the EGF receptor. Further study of the function of RPTP\(\beta\) in target cells may well provide further information concerning the mechanism of VacA toxicity on host cells.

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