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Helicobacter pylori Vacuolating Cytotoxin Binds to the 140-kDa Protein in Human Gastric Cancer Cell Lines, AZ-521 and AGS

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To investigatie a potential mechanism of how *Helicobacter pylori* establishes infection, we purified a lot of vacuolating toxin (VacA) from supernatant of *H. pylori* ATCC49503 (tox$^+$ strain 60190). We used an antibody which was prepared by immunizing rabbits with a synthetic peptide consisting of 16 amino acids reflecting a portion (Glu^{69}-Arg^{83}) of amino acid sequence of Vac A. VacA caused vacuoles in human gastric cancer cell lines AZ-521 AGS, and monkey kidney cell line COS-7, but not human promyeloblastic cell line HL-60. By immunoprecipitation analysis using anti VacA antibody, a biotinylated cell surface protein of 140kDa (p140) was precipitated only when the lysates of VacA-susceptible cells were incubated with VacA but not with inactivated VacA, indicating the association of p140 with VacA. © 1997 Academic Press

Helicobacter pylori is an etiologic agent of gastritis, gastric ulcer, and gastric cancer. The vacuolating toxin (VacA) is the cytotoxin produced in vitro by about 50% of *H. pylori* and causes vacuolation in various cells. VacA acts as an oligomeric toxin which consists of 5 or 6 molecules of a 87-95 kDa mature toxin released after proteolytic cleavage of its 140kDa precursor at the bacterial outer membrane. A 58kDa C-terminal fragment of VacA is thought to mediate cell surface binding[1)]. VacA induces large vacuoles enriched with a small GTP binding protein, rab7, by interaction with membrane and/or intracellular component[2)]. There are many reports showing that the the receptor molecules for bacterial cytotoxins are located in the membrane surface of their target cells. Determination of the receptor molecule is not only crucial in the understanding of the pathogenesis of a particular toxin but it may also have an application in the detoxification process involving life threatening bacterial infections (e.g. verotoxin receptor analogs are used to absorb the free toxin in the intestinal tract before it can be absorbed). However, little is known about the receptor molecule for VacA.

Here we report that a cell surface protein (p140) with a molecular mass of 140kDa can bind to VacA. This p140 was distributed in VacA susceptible cells including human gastric cancer cell lines, AZ-521 and AGS, and the monkey kidney cell line, COS-7, but not in the human promyeloblastic cell line, HL-60, which is insensitive to VacA.

MATERIALS AND METHODS

Bacterial strain and toxin production. The toxin-producing strain *H. pylori* ATCC49503 (tox$^+$ strain 60190) was used as the source for purification of Vac A. Colonies of *H. pylori* grown on a blood agar plate (Brucella agar with 5 % horse blood) were inoculated into Brucella broth containing 0.1% β-cyclodextran and were cultured at 37°C for 3-4 days with vigorous shaking in a controlled micro-aerophilic atmosphere of 10% O_2 and 10% CO_2 generated with a aero pack (Mitubishi Gas Chemicals Co., Osaka).

Preparation of purified VacA. The culture was centrifuged at 6000 × g for 15 min, and the supernatant was collected. The proteins present in the supernatant were precipitated with 50% saturated ammonium sulfate. After centrifugation at 12,000 × g for 30 min, the resulting precipitate was suspended in 10 mM phosphate buffer, pH 7.2, containing 1 mM PMSF and dialyzed overnight against the same buffer. The dialyzed sample was applied to a Econo-Pac cartridge CHT-II column (Bio-Rad), hydroxyapatite (1.2×3.5 cm), equilibrated with the buffer containing 10 mM phosphate buffer, pH 7.2. After washing the column with the same buffer, Vac A was eluted with a linear gradient of 10 mM to 360 mM. Fractions containing

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Abbreviations used: SDS–PAGE, SDS-polyacrylamide gel electrophoresis; BSA, bovine serum albumin; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; D-MEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; KLH, Keyhole limpets hemocyanin.

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VacA that formed a reactive band with an antibody, which was prepared by immunizing rabbits with a synthetic peptide consisting of 16 amino acids reflecting a portion (Glu^{69}-Arg^{83}) of amino acid sequence of Vac A[3], were collected and solid ammonium sulfate was added to the pooled material at 4°C to 50% saturation. The precipitate was collected by centrifugation at 12,000 × g for 30 min, dissolved in 1ml of 60 mM Tris-HCl buffer, pH 7.7, containing 0.1M NaCl, and subjected to FLPC on a Superose 6HR 10/30 column (1 × 30 cm). The column was equilibrated with 60 mM Tris-HCl buffer, pH 7.7, containing 0.1M NaCl and the material was eluted with the same buffer at the flow rate of 0.5 ml/min. The eluted protein and VacA were monitored for UV absorbance at 280 nm and Western blotting using the anti VacA-peptide antibody. The fractions containing VacA were collected, pooled, diluted with deionized water to 3-fold, and subjected to an anion exchange chromatography. Anion exchange chromatography was performed on a Resource Q column (Pharmacia, 0.64 × 3 cm) with 20 mM Tris-HCl buffer, pH 7.7. The sample was eluted with a linear gradient of 0.05-0.6 M NaCl in the same buffer. The fractions were concentrated by Centriplus concentrator with Amicon Centriplus-100. Finally, the concentrated material was applied again to Superose 6 HR 10/30 column under the same condition as described above.

Assay of vacuolating activity. Human gastric adenocarcinoma cell line AZ-521 (Culture Collection of Health Science Research Resources Bank, Japan Health Sciences Foundation) was seeded in D-MEM supplemented with 10% FCS in 96-well tissue culture plates at 1×10^5 cells per well in a volume of 80 ul per well and cultured as monolayers in the plates for 24 h in a 5% CO_2 atmosphere at 37°C. Twenty microliter aliquots of VacA were added at a concentration of 10 ug/ml and cells were incubated for an additional 24 h at 37°C. To quantify the vacuolating activity, the uptake of neutral red into the vacuoles in the treated cells with VacA was determined as reported by Cover, T.L. et al[4]. The treated cells were incubated for 5 min at room temperature with 50 ul of freshly prepared 0.05% neutral red in PBS containing 0.3% BSA, and then washed 3-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 (OD_{540}) was measured. Vacuolating activity was determined by subtracting the OD_{540} of cells incubated without VacA from the OD_{540} of VacA treated cells.

Heat-stability of vacuolating activity of VacA (1 ug) was tested after incubation at various temperatures for 10 min, and followed by assay of its remaining activity.

Preparation of anti peptide and anti VacA antibodies. Rabbits were immunized according to the following schedule: first, a synthetic peptide-coupled KLH (200 mg) or a purified Vac A (40 ug) in complete Freund's adjuvant (1:1) subcutaneously on day 0; second, 200 mg of the peptide-coupled KLH or 40 ug of VacA in incomplete Freund's adjuvant (1:1) subcutaneously on day 14; and finally, 200 mg of the peptide antigen or 40 ug of Vac A with 4 mg alum intraperitoneally on day 21. Animals were bled 4 and 5 weeks after the first injection.

Immunoprecipitations. The cultured cells were harvested with TNE buffer (1.5 ml), containing 40 mM Tris-HCl (pH7.5), 150 mM NaCl, and 1 mM EDTA, and washed twice with PBS. The protein localized on the cell surface was biotinylated according to the manufacture's specifications (Amersham; ECL Protein biotinylation module). Biotinylated cells were lysed with 0.5 ml of Sol buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10% glycerol, 10ug of leupeptin per ml, and 1% Triton X-100) for 10 min on ice. After centrifugation for 20 min at 100,000 × g, the supernatant (100 ul) was exposed to 20 ug of VacA or an inactivated VacA, which was prepared by 95°C-treatment for 10min, at 37°C for 1h and then incubated with 1 ul of an antibody raised against purified VacA or a preimmune rabbit serum as a control at 4°C overnight. Antibody-bound proteins were collected by addition of 40 ul of a 0.1-g/ml protein A Sepharose CL-4B (Pharmacia) solution in Sol buffer at 4°C for 1 h. Immunoprecipitates were washed four times with Sol buffer containing 1% sodium deoxycholate and 0.1% SDS, solubilized by heating with 0.1ml of SDS-PAGE sample buffer containing 50mM Tris-HCl (pH6.8), 1.6% SDS, 8% glycerol, 2% dithiothreitol, and 0.08% bromphenol blue, and separated on a SDS-7.5% polyacrylamide gel by the method of Laemmli[5]. For immunoblotting, the proteins were transferred to PVDF membrane (Millipore; Immobilon-P membranes) at 100 V for 1 h at 4°C with Western transfer buffer (25mM Tris, 0.19M Glycine, 20% Methanol). Membranes were blocked with 0.5 % milk powder in PBS containing 0.1% Tween 20 for 30 min, and washed in PBS containing 0.1% Tween 20 without milk. After extensive washing with PBS, the immunoprecipitated proteins were detected with horseradish peroxidase-conjugated streptavidin followed by enhanced chemiluminescence (ECL) detection (Amersham) as described in previous paper[6].

Other methods and chemicals. Protein was measured by the method of Bradford using bovine serum albumin as a standard. A ganglioside mixture prepared from mouse brains was a gift from S. Kosaki (College of Agriculture, University of Osaka Prefecture, Japan)[7] and its quantity was defined by the amount of NeuAc as determined by Svennerholm[8]. The molecular mass standards used were phosphorylase b (cross-linked trimer; 292kDa), myosin (220kDa), rabbit muscle phosphorylase b (97.4 kDa), BSA (66kDa), hen egg albumin (46kDa), and carbonic anhydrase (31kDa). β-Cyclodextran was kindly offered from Teijin (Tokyo, Japan). Other reagents were of analytical grade.

RESULTS AND DISCUSSION

Characterization of Purified VacA

To investigate a potential mechanism of how *H. pylori* establishes infection, we purified a lot of VacA from a supernatant of *H. pylori* ATCC49503 using a method previosly described by T.L Cover and M.J. Blaser[9]. After VacA present in the culture supernatant of *H. pylori* was precipitated and purified, it was subjected to SDS-PAGE analysis. When 1 μg of the purified VacA was applied, VacA appeared a single band stained with Coomassie Brilliant Blue at the position of 87kDa on a 10% SDS-PAGE gel **(Fig. 1A)** and was detected as a single band reacted with an anti-VacA antibody **(Fig. 1B).** As previously shown by Cover, T.L. et al[10], the rapid neutral red uptake assay method is useful to quantitate vacuolation of the cells exposed to VacA. We employed this method for testing the toxin susceptibilities of four cell lines, the human gastric cancer cell lines, AZ-521 and AGS, monkey kidney cell line, COS-7, human leukemia cell line, HL-60, to VacA. The result **(Fig. 2)** shows that dose-dependent vacuolation induced by VacA was formed in AZ-521, AGS and COS-7, but not in HL-60. AZ-521 is distinguished by its high sensitivity to VacA, resulting in formation of many vacuoles with incorporated dye in the cytosolic spaces; therefore this cell line was chosen for further experiments. The minimum dose of VacA to cause vacuoles in AZ-521 (1×10^5 cells) at 24h-incubation was 0.25 ug and an exposure to over 3 μg of VacA resulted in cell destruction (data not shown). Vacuolating activity of 1 μg of VacA was completely diminished and neutralized with anti-VacA antibody. When VacA was incubated

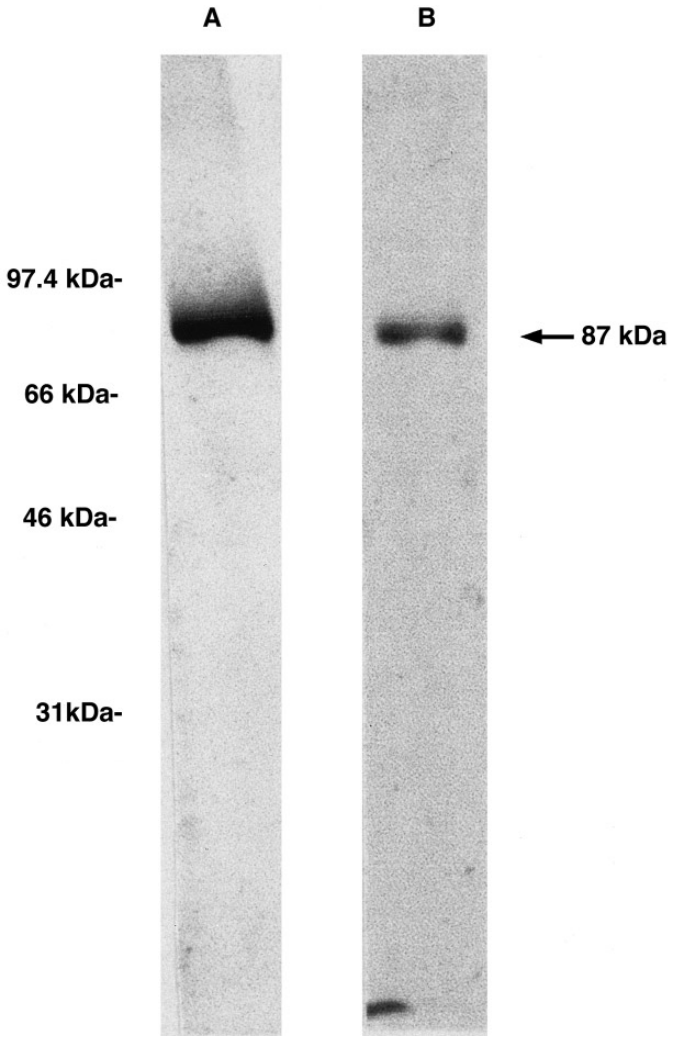

FIG. 1. SDS–PAGE showing a purified VacA with silver staining (A) and its Western blot visualized with anti VacA antibody (B). VacA (1 μg) was subjected to SDS–PAGE with a 10% separating gel. Molecular mass standards are shown on the left. The arrow at 87kDa shows VacA.

at various temperatures for 10 min, its activity was stable up to 55°C but high temperatures above 65°C resulted in marked loss of activity **(Fig. 3)**.

Immunoprecipitation of Vac A Receptor Protein

We tested and found that the pretreatment of VacA with the ganglioside mixture (50 μg/ml of NeuAc) prepared from mouse brains did not have any effect on vacuolating activity for AZ-521 cells (data not shown). To determine whether there was a protein receptor for VacA on the cell surface, immuoprecipitarion analysis of biotinylated cell surface proteins in susceptible cells and nonsusceptible cell was performed in the presence of VacA or heated VacA using anti-VacA antibody as described in the text **(Fig. 4).** By Western blotting, an

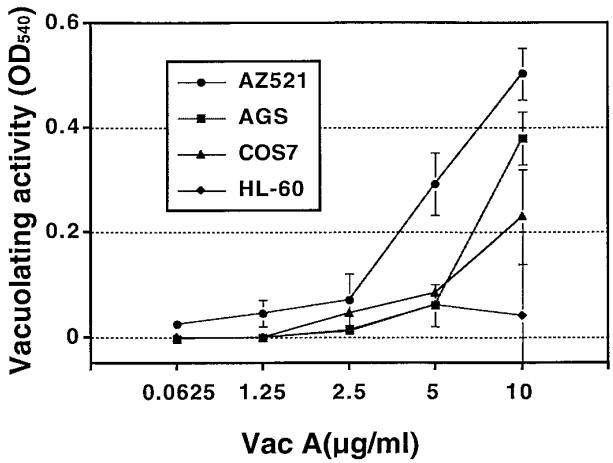

FIG. 2. Induction of vacuolation in various cells by VacA. Various cells (10^5 cells) such as AZ-521 (●), AGS (■), COS-7 (▲), and HL-60 (♦) were incubated with the indicated amounts of VacA for 24 h. Vacuolating activity was determined by neutral red up take assay. Error bars indicate the standard deviations of the means for three separate experiments.

intense band at a position of 140kDa was commonly observed only when VacA susceptible cells such as AZ-521, AGS, and COS-7 were subjected to this experiment with VacA, but not in case of HL-60, which is a nonsusceptible cell for VacA **(Fig. 2).** Two high molecular mass bands of about 250 kDa and150kDa were also detectable for AZ-521 and COS-7, respectively. The other protein bands can be attributed to their nonspe-

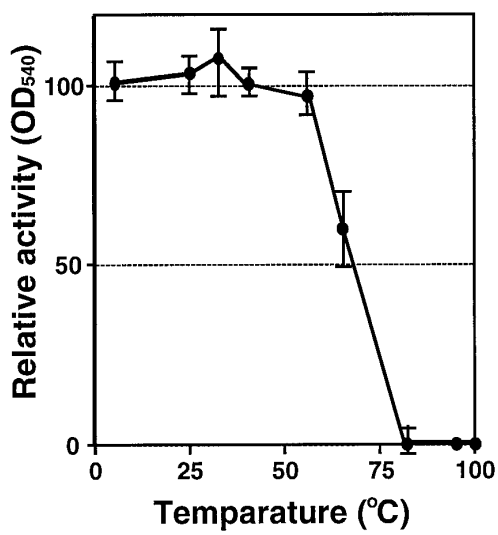

FIG. 3. Heat stability of vacuolating activity of VacA. After treatment of VacA (10 μg/ml) at indicated temperature for 10 min, the remaining vacuolating activity was determined under standard assay conditions. Values for remaining activity of VacA are shown as percentage of that obtained by treatment at 4°C. Error bars indicate the standard deviations of the means for three separate experiments.

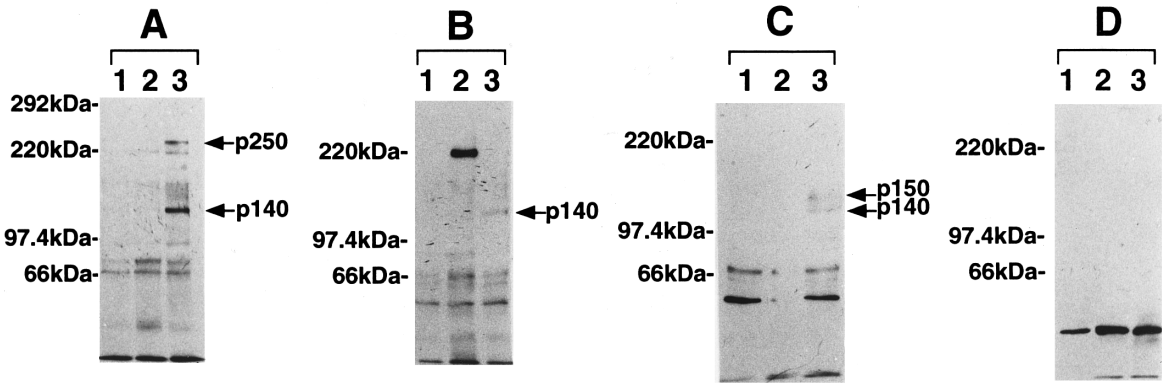

FIG. 4. Immunoprecipitation analysis of biothinylated VacA-binding protein on various cells. After biotinylation of cell surface proteins of AZ-521 (A), AGS (B), COS-7 (C), and HL-60 (D), VacA (18 μg/ml) or an inactivated VacA was added to the cell lysates, prior to the immunoprecipitation of VacA binding protein with anti VacA antibody and the protein A Sepharose CL-4B. Lanes: 1, control (buffer alone); 2, an inactivated VacA; 3, VacA. The immunoprecipitated proteins were detected with horseradish peroxidase-conjugated streptavidin followed by ECL detection. The migration position of molecular mass standards (in kilodaltons) are noted on the left.

cific interaction with protein A-Sepharose CL-4B, since these protein bands were detected when protein A-Sepharose CL-4B and the cell lysates were mixed in the absence of the anti VacA antibody or an inactivated VacA, respectively (data not shown). Thus, these results indicate that a novel p140 on the cell surface can bind and associate with VacA. It is possible that this interaction is an initial step in internalization of VacA into the cells through endocytosis process[11] or a signaling pathway that mediate pepsinogen secretion at gastric tissues[12].

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