Protein-tyrosine Phosphatase α, RPTPα, Is a Helicobacter pylori VacA Receptor*

Received for publication, January 6, 2003, and in revised form, March 5, 2003
Published, JBC Papers in Press, March 6, 2003, DOI 10.1074/jbc.M300117200

Kinnosuke Yahiro*, Akihiro Wada‡‡, Masaaki Nakayama*, Takahiro Kimura*, Ken-ichi Ogushi‡, Takuro Niidome†, Haruhiko Aoyagi†, Ken-ichi Yoshino‡, Kazuyoshi Yonezawa‡, Joel Moss**, and Toshiya Hirayama‡ ‡‡

From the *Department of Bacteriology, Institute of Tropical Medicine, Nagasaki University, Nagasaki 8528523, Japan, §PRESTO, Japan Science and Technology Corporation, Saitama, Japan, the ¶Department of Applied Chemistry, Faculty of Engineering, Nagasaki University, Nagasaki 8528521, Japan, the ¶¶Biological Research Center, Kobe University, Kobe 6578501, Japan, and the **Pulmonary-Critical Care Medicine Branch, NHLBI, National Institutes of Health, Bethesda, Maryland 20892-1590

Helicobacter pylori vacuolating cytotoxin, VacA, induces vacuolation, mitochondrial damage, cytochrome c release, and apoptosis of gastric epithelial cells. To detect gastric proteins that serve as VacA receptors, we used VacA co-immunoprecipitation techniques following biotinylation of the cell surface and identified p250, a receptor-like protein-tyrosine phosphatase (RPTPα) as a VacA-binding protein (Yahiro, K., Niidome, T., Kimura, M., Hatakeyama, T., Aoyagi, H., Kurazono, H., Imagawa, K., Wada, A., Moss, J., and Hirayama, T. (1999) J. Biol. Chem. 274, 36693–36699). VacA causes vacuolation of G401 cells, a human kidney tumor cell line, although they do not express RPTPα. By co-immunoprecipitation with VacA, we identified p140 as a potential receptor in those cells. p140 purified by chromatography on a peanut agglutinin affinity matrix contained internal amino acid sequences of RGEENTDYVNAS and AEGILDVFGTQVK, which are identical to those in RPTPα. The peptide mass fingerprinting of p140 by time of flight-MS analysis also supported this identification. Treatment of G401 cells with RPTPα-morpholino antisense oligonucleotide before exposure to toxin inhibited vacuolation. These data suggest that RPTPα acts as a receptor for VacA in G401 cells. Thus, two receptor tyrosine phosphatases, RPTPα and RPTPβ, serve as VacA receptors.

Persistent infection with Helicobacter pylori causes chronic active gastritis, which predisposes the mucosa to peptic ulceration, and is believed to participate in the pathogenesis of gastric carcinoma and primary gastric lymphoma (i.e. mucosa-associated lymphoid tissue, or MALT type) (1, 2). Although H. pylori is a noninvasive bacterium that survives in the stomach mucosa, pathogenic strains of H. pylori produce and secrete a potent vacuolating cytotoxin, VacA. Both epidemiological study (3) and animal experiments (4–7) have demonstrated that VacA is a major virulence factor and is involved in the pathogenesis of inflammation in H. pylori-induced gastritis and ulceration. Oral administration of VacA to mice caused acute inflammation of the gastric mucosa with accumulation of mast cells, the activation of which by VacA resulted in production of proinflammatory cytokines (8). Intense infiltration of granulocytes and lymphocytes was observed following mast cell activation, consistent with the hypothesis that mast cells contribute to the gastric inflammation in H. pylori-infected peptic gastritis and ulceration (9).

Purified VacA has a molecular mass of 87–95 kDa under denaturing conditions, whereas the native toxin is an oligomeric complex of about 1000 kDa (10). VacA proved to be capable of directly inducing progressive vacuolation (11), mitochondrial damage (12), cytochrome c release (13), and apoptosis of epithelial cells (14). Although the detailed mechanism of its toxic activity is still unknown, baflomycin A1, a specific inhibitor of the vacular ATPase proton pump, can inhibit VacA-induced vacuolation. It did not, however, block mitochondrial damage by VacA (12). In addition, a mutant VacA lacking vacuolating activity induced an apoptosis of AGS cells (14). These results led to the current model in which the mitochondrial damage occurs via a pathway different from vacuolation induced by VacA, possibly through the formation of pores or selective anion channels in endosomal membranes (15, 16) and alteration of endolysosomal function (17, 18).

VacA binding to specific high-affinity cell-surface receptors was shown by using indirect immunofluorescence and flow cytometry; high-affinity toxin binding was necessary for cell intoxication (19, 20). VacA can interact with target cells by binding to receptor-like protein-tyrosine phosphatase β (RPTPβ) (1, 21). In addition to RPTPβ, other proteins of lesser abundance on the surface of AZ-521 cells were also immunoprecipitated with anti-VacA antibody (22), consistent with the presence of alternative receptors.

G401 cells, although responsive to VacA, do not express RPTPβ. Here we report purification of another VacA receptor, p140, and its identification as the receptor-like tyrosine phosphatase α, RPTPα, a ubiquitously expressed protein (24). Thus, RPTPα and RPTPβ, two receptor-like protein-tyrosine phosphatases that differ widely in structure, can both serve as VacA receptors.

* This work was supported by grants-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, the Naito Foundation, and the Uehara Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡‡ To whom correspondence should be addressed: Dept. of Bacteriology, Institute of Tropical Medicine, Nagasaki University, Nagasaki 852-8523, Japan. Tel.: 81-95-849-7831; Fax: 81-95-849-7805; E-mail: hirayama@net.nagasaki-u.ac.jp.

1 The abbreviations used are: RPTP, receptor-like protein-tyrosine phosphatase; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; EPEI, ethoxylated polyethylenimine; FCS, fetal calf serum; HRP, horseradish peroxidase; MAA, Maackia amurensis agglutinin; MES, 2-mercaptoethane sulphonic acid; NRU, neutral red uptake; PNA, peanut agglutinin; PVD, polyvinylidene difluoride; PBS, phosphate-buffered saline.
Materials and Methods

Cell Culture—G401, Wilms' human kidney tumor cells (ATCC CRL-1441), and COS-7 cells were grown in DMEM (Sigma) containing 10% FCS. HL-60 cells were cultured in RPMI 1640 medium with 20 nm phorbol 12-myristate-13-acetate to stimulate differentiation into macrophage-like cells (22).

VacA Preparation—The toxin-producing H. pylori strain ATCC49503 was used as the source of VacA for purification according to a modified published procedure (23). Briefly, 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 microaerophilic atmosphere of 10% O2 and 10% CO2, VacA was precipitated from culture supernatant with 50% saturated ammonium sulfate and purified by affinity column chromatography. Ammonium sulfate precipitates were dialyzed against RX buffer (10 mM KCl, 0.3 mM NaCl, 0.53 mM MgCl2, and 0.125 mM EGTA in 1 mM HEPES, pH 7.3) and applied to an anti-VacA-specific IgG antibody column (10 mg of IgG2 mg of dried resin) equilibrated with RX buffer. After washing the column with RX buffer, VacA was eluted with 50 mM glycine-HCl buffer (pH 1.0), which was subsequently neutralized with 1 M Tris-HCl (pH 10). After gel filtration on Superose 6HR 10/30 equilibrated with TBS buffer (60 mM Tris-HCl buffer, pH 7.7, containing 0.1 M NaCl, and 1 mM EDTA), and washed twice with PBS. After bioaffinity chromatography, proteins were eluted with 50 mM glycine-HCl buffer (pH 1.0), which was subsequently neutralized with 1 M Tris-HCl (pH 10). After gel filtration on Superose 6HR 10/30 equilibrated with TBS buffer (60 mM Tris-HCl buffer, pH 7.7, containing 0.1 M NaCl), purified VacA was concentrated and stored (200 μg/ml).

Immunoprecipitation of p140—Immunoprecipitation of VacA receptors was performed as previously described (21). In brief, G401 cells were harvested in TNE buffer (40 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, and 1 mM EDTA), and washed twice with PBS. After bioaffinity chromatography, proteins were eluted with 50 mM glycine-HCl buffer (pH 1.0), which was subsequently neutralized with 1 M Tris-HCl (pH 10). After gel filtration on Superose 6HR 10/30 equilibrated with TBS buffer (60 mM Tris-HCl buffer, pH 7.7, containing 0.1 M NaCl), purified VacA was concentrated and stored (200 μg/ml).

Characterization of Carbohydrate Moieties of p140—Specific binding of p140 to cells was identified by carbohydrate moieties of p140 from G401 cells, according to the manufacturer's specifications (Roche Diagnostics; DIG glycans differentiation kit) as previously described (21). The lectins selectively recognize terminal sugars; MAA and PNA recognize sialic acid terminal linked α2–3) to galactose and galactose-β-(1–3)-N-acetylgalactosamine, respectively. PVDF membranes with proteins that had been separated by SDS-PAGE after immunoprecipitation were incubated at 25 °C for 1 h with lectin conjugated to the steroid hapten digoxigenin. After washing the membrane, glycoproteins were incubated with anti-digoxigenin Fab fragments conjugated with alkaline phosphatase, followed by detection with 4-nitro blue tetrazolium chloride/β-bromo-4-chloro-3-indolyl phosphate.

Analysis of p140—To examine binding of p140 to PNA-agarose (5 μl bed volume), glycoproteins (200 ng) were added to a mixture of 34.5 μl (1 mg/ml) of biotinylated p140. The column was washed with 25 μl of Sol buffer, and then Sol buffer containing 0.3 mg/ml-galactose was used to elute the carbohydrate-containing proteins in 1-ml fractions. After SDS-PAGE in 5% gels, proteins in eluates were transferred to PVDF membranes, detected using enhanced ECL.

Fluor Cytometric Analysis—AZ-521 or G401 cells (1 × 107) were harvested in TNE buffer, washed twice in PBS, and suspended in 1 ml of PBS containing 2% BSA. Samples (90 μl) were treated with neuraminidase (0.1 unit) for the indicated times. After two washes with PBS containing 2% BSA, cells (1 × 107/90 μl) were incubated at 4 °C for 30 min with 10 μl (1 μg/ml) of VacA or PBS as a control. Cells were washed twice with 500 μl of PBS containing 2% BSA, followed by addition of anti-VacA antibody and incubation at 4 °C for 30 min. After two washes with PBS containing 2% BSA, cells were suspended in 400 μl of the same solution containing Fluoro Light ™ Cy™2-labeled goat anti-rabbit IgG (1:400) (Amersham Biosciences) and incubated at 4 °C for 30 min. After two washes with PBS containing 2% BSA, cells were suspended in 300 μl of PBS containing 2% BSA and incubated with 120 μl of native VacA at 4 °C for 30 min, before washing twice with 500 μl of PBS containing 2% BSA and addition of 300 μl of DMEM containing 10% FCS. Samples (100 μl) were incubated in 96-well plates for 3 h at 37 °C and vacculat- ing activity was quantified by NRU assay.

Immunoprecipitation of p140 was performed using the Ribonuclease Protection Assay kit (RPAII; Ambion) as previously described (22). Briefly, 20 μg of total RNA from G401 cells, positive control cells such as AZ-521 cells, and HL-60 cells treated with 20 nm phorbol 12-myristate-13-acetate for 48 h were used in the RNase A protection assay (22). Total RNA was hybridized overnight with RPTPβ and glyceraldehyde-3-phosphate dehydrogenase probes in hybridization buffer at 42 °C, followed by treatment with RNase, which was then inactivated. Protected fragments were separated in 5% sequencing gels, and quantified by Fuji film autoradiography. Sizes of protected fragments were 250 nucleotides for RPTPβ and 184 nucleotides for glyceraldehyde-3-phosphate dehydrogenase.

Assay for Vacuolating Activity—Vacuolating activity was assayed using AZ-521 or G401 cells as previously described (21). Cells (1 × 105 cells/well, 250 μl) were grown in 24-well culture plates as monolayers for 24 h in a 5% CO2 atmosphere at 37 °C. VacA was added and cells were incubated for an additional 8 h at 37 °C. To quantify the vacuolating activity, the uptake of neutral red into the vacuoles was determined as described previously (21).

Glycosidase Treatment—After purification of proteins on the surface, G401 cells were lysed in 1 ml of Sol buffer for 15 min on ice. After centrifugation (20 min, 15,000 × g), 10 μl of each supernatant (1 μg/ml) was added to a mixture of 34.5 μl of 20 mM sodium phosphate buffer (pH 7.2), 2.5 μl of 10% Nodul-P-40, and 0.5 μl of N-glycosidase F (0.1 unit), neuraminidase (0.1 unit), heparitinase (0.5 unit), or chondroitinase ABC (0.5 unit), followed by incubation for 3 h, and immunoprecipitation with anti-VacA after addition of native VacA or a heat-inactivated VacA. Immunoprecipitated proteins were separated by SDS-PAGE in 5% gels and transferred to PVDF membranes.

To examine binding of p140 to PNA-agarose (5 μl bed volume), glycoproteins (200 ng) were added to a mixture of 34.5 μl (1 mg/ml) of biotinylated p140. The column was washed with 25 μl of Sol buffer, and then Sol buffer containing 0.3 mg/ml-galactose was used to elute the carbohydrate-containing proteins in 1-ml fractions. After SDS-PAGE in 5% gels, proteins in eluates were transferred to PVDF membranes, detected using enhanced ECL.
were detected using the ECL system (Amersham Biosciences). An anti-V5 monoclonal antibody (Invitrogen), which recognizes the 14-amino acid (GKP1PNPLLGLDST) sequence of V5 (28), and proteins were detected using the ECL system (Amersham Biosciences).

Transfection of G401 Cells with RPTPα-morpholino Antisense Oligonucleotide—RPTPα-morpholino antisense oligonucleotide (5'-GAA-CAGAATGAGCAGAATCATC-3') was designed and purchased from Gene Tools, LLC (Philomath, Oregon). G401 cells were seeded in 24-well culture plates (5 × 10⁴ cells in 1 ml of DMEM per well) and incubated overnight at 37 °C. The gene delivery agent EPEI and 1.4 μM RPTPα-morpholino antisense oligonucleotide were incubated in 500 μl of FCS-free DMEM for 20 min at room temperature before addition to G401 cells in 24-well plates, followed by incubation for 3 h at 37 °C. After the culture medium was replaced with 500 μl of DMEM containing 10% FCS, cells were incubated for 16 h. Vaccination of cells caused by incubation with 120 μl VacA for 0, 2, and 4 h was quantified by NRU.

**RESULTS**

Identification of p140 on G401 Cells—The Wilms’ tumor cell line G401 was susceptible to VacA intoxication (Fig. 1). In Western blot analysis using anti-RPTPα antibody, RPTPα was, however, not detected in G401 cell lysates; the lack of RPTPα expression in G401 cells was confirmed by RNase protection assays (Fig. 2A). Thus, G401 cells did not express RPTPα protein or mRNA as suggested by Qi et al. (28) who used reverse transcriptase-PCR. A 140-kDa protein on the surface of G401 cells, however, was immunoprecipitated with anti-VacA antibody and protein A-Sepharose CL-4B after incubating the...
cell lysate with VacA, but not with inactivated VacA (Fig. 2B). These results were consistent with the hypothesis that not only RPTPα/H9251, but also p140, can be a functional VacA receptor and led us to use G401 cells for purification of p140.

Characteristics of Sugar Moiety of p140—PNA and MAA bound p140 (Fig. 3) through interaction with terminal sialic acid linked to (2–3)–galactose and galactose–(1–3)–N-acetylgalactosamine, respectively. After incubation with glycosidases, biotinylated p140 on G401 cells was subjected to immunoprecipitation with anti-VacA antibodies after incubation with native VacA or heat-inactivated VacA (Fig. 4A). The samples immunoprecipitated via their association with native VacA before (Fig. 4A, lane 2) and after (Fig. 4A, lane 4) N-glycosidase F treatment contained 140- and 135-kDa proteins, respectively. No significant change in molecular size of p140 was observed after treatment with chondroitinase ABC, or heparitinase. As shown in lane 8 (Fig. 4B), neuraminidase treatment inhibited p140 binding to VacA, an effect confirmed by flow cytometric analysis of VacA binding to G401 cells treated with neuraminidase (Fig. 5). Treatment of G401 cells with neuraminidase before incubation with VacA decreased fluorescence intensity to control levels. In addition, two kinds of sialyllactosamine with terminal sialic acids, 3'-sialyllactosamine and 6'-sialyllactosamine, did not interfere with VacA binding to p140 when the biotinylated p140 on G401 cells was immunoprecipitated using native VacA and anti-VacA antibody (data not shown). These results indicate that not only the

**Fig. 3. Lectin blot analysis of p140 in G401 cells.** 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. MAA- and PNA-binding proteins (panels A and B), detected by reaction with lectin conjugated to digoxigenin followed by incubation with antidigoxigenin 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 p140 immunoprecipitated with anti-VacA antibody, separated by SDS-PAGE in 5% gels, transferred to PVDF membrane, and incubated with HRP-conjugated streptavidin followed by ECL detection. Data are representative of three separate experiments.

**Fig. 4. Immunoprecipitation of p140 after treatments with N-glycosidase F, neuraminidase, chondroitinase ABC, and heparitinase.** Proteins solubilized from biotinylated cells were incubated without (lanes 1, 2, 5, and 6) or with N-glycosidase F (N-gly F, lanes 3 and 4; panel a), neuraminidase (neura, lanes 7 and 8), chondroitinase ABC (chondo, lanes 9 and 10), or heparitinase (hepa, lanes 11 and 12) (panel b) at 37 °C for 3 h. Samples were then incubated with heat-inactivated VacA (I) or native VacA (A) before immunoprecipitation with anti-VacA antibodies and SDS-PAGE in 5% gels followed by transfer to PVDF membranes; p140 was visualized by incubation with HRP-conjugated streptavidin followed by ECL detection. Arrows show the location of p140. Data are representative of three separate experiments.

**Fig. 5. Effect of neuraminidase treatment of AZ-521 cells and G401 cells on VacA binding to the cells.** Cells were incubated with neuraminidase for 0 (f), 0.5 (e), 1 (d), or 1.5 h (c). The treated cells were incubated with VacA for 30 min, then incubated with anti-VacA antibody for 30 min, and finally for 30 min with Fluoro Link™ Cy™2-labeled goat anti-rabbit IgG, followed by FACS result. The results are plotted as relative cell number versus Fluoro Link™ Cy™2 fluorescence and are representative of three experiments; 10,000 cells were analyzed per sample. Control (b) was prepared without VacA after incubation with neuraminidase for 1.5 h. Another control (a) was prepared without treatment with neuraminidase and VacA. Data are representative of three separate experiments.
terminal sialic acid but also specific sugar structure and p140 sequence may be required for VacA binding.

Inhibition of VacA-induced Vacuolation in AZ-521 and G401 Cells by Neuraminidase Treatment—As treatment of AZ-521 and G401 cells with neuraminidase diminished the binding of VacA, we examined whether neuraminidase treatment also diminished VacA-induced vacuolation (Fig. 6). VacA-induced vacuolation of both AZ-521 and G401 cells treated with neuraminidase was found to be significantly less than that of untreated cells.

Purification and Sequence Analysis of p140—By using PNA lectin affinity chromatography with solubilized G401 cells, we obtained partially purified p140 from fractions (E1–3) eluted with Sol buffer containing 0.3 M d-galactose (Fig. 7). Proteins in fractions, in which p140 was detected by immunoprecipitation (Fig. 7a), were precipitated with 10% trichloroacetic acid, solubilized with SDS-PAGE sample buffer, and separated in 5% gels, which were stained with Coomassie Brilliant Blue (Fig. 7b). Internal sequences obtained from two peptides separated by SDS-PAGE after Achromobacter protease I hydrolysis of p140 and excised from the gel were RGEENTDYVNASFIDGY-RQK and AEGILDVFGQTVK, which are identical to positions 581–600 and 754–765 in the RPTPα sequence reported by Kaplan et al. (29). In agreement with these data, peptide mass fingerprinting of p140 by time of flight-MS analysis after proteolysis (25) showed that all fragments were identical to those predicted for RPTPα (data not shown). Thus, these analytical data lead to the conclusion that p140 is RPTPα.

VacA Binding to RPTPα in RPTPα Gene-transfected COS-7 Cells—Using COS-7 cells, which we transfected with V5-tagged RPTPα cDNA with relatively high efficiency, we examined the relationship of RPTPα expression to VacA binding; of note, COS-7 cells are sensitive to VacA and exhibit baseline VacA binding (23). COS-7 cells transfected with V5-tagged RPTPα cDNA exhibited greater VacA binding than did untransfected cells or cells transfected with vector only. Overexpression of V5-tagged RPTPα in the cell lysate or cell membrane was confirmed by visualization using Western blotting with HRP-conjugated anti-V5 antibody and its binding activity was assayed by immunoprecipitation using VacA and anti-VacA antibody, followed by visualization of V5-tagged RPTPα in immunoprecipitates (Fig. 8). Anti-V5 antibody recognized two expressed V5-tagged RPTPα products of ∼140 and 100 kDa in lysates of transfected cells (Fig. 8b). Using immunoprecipitation analysis, however, only the 140-kDa form could apparently bind VacA (Fig. 8c). To characterize the effects of VacA in COS-7 cells expressing RPTPα, we assayed VacA-induced vacuolation in cells transfected with vector or RPTPα gene after incubation with 120 nM VacA for 8 h (data not shown). Both untransfected and transfected cells with the same magnitude of the vacuole formation in transfected cells were slightly increased by treatment with VacA, indicating that additional binding of VacA did not result in a remarkable increase in vacuoles in RPTPα-expressing cells.

RPTPα Antisense Oligonucleotide—To examine the relationship between VacA sensitivity and RPTPα expression in G401 cells, cells were treated with 1.4 μM RPTPα-morpholino antisense oligonucleotide followed by incubation with 120 nM VacA.
In transfected COS-7 cells, two forms of overexpressed V5-tagged RPTPα have higher molecular masses (140 and 110 kDa) than the mass calculated (90,559) from RPTPα cDNA (29), suggesting that they have undergone glycosylation. Interestingly, V5-tagged RPTPα of 140 kDa, but not the 110-kDa form, bound VacA, suggesting that the sugar modification of RPTPα is important for binding to VacA. In agreement with this suggestion, neuraminidase treatment diminished the binding to VacA and VacA-induced vacuolation as shown in Figs. 5 and 6.

For many bacterial toxins, interaction with specific cell-surface receptors results in toxin internalization. VacA is known to be internalized by eukaryotic cells (19, 31), and several lines of evidence indicate that VacA-induced cell vacuolation results from VacA activity at an intracellular site (17, 18, 32). Fig. 10 shows that RPTPα functions as a receptor for VacA on AZ-521 cells, a human gastric cancer cell line (23, 24); a second protein, p140, was also commonly detected in AZ-521 and AGS, including non-gastric VacA-sensitive cells such as the monkey kidney line COS-7 (19). Here we report that internal amino acid sequencing non-gastric VacA-sensitive cells such as the monkey kidney line COS-7 (19). Here we report that internal amino acid sequence analysis and time of flight-mass analysis of p140, which was isolated from G401 cells, identified their receptor as RPTPα. In agreement with this finding, the binding of V5-tagged RPTPα to VacA was demonstrated by immunoprecipitation using VacA and anti-VacA antibody as shown in Fig. 8. In addition, treatment of G401 cells with RPTPα-morpholino antisense oligonucleotide before exposure to VacA inhibited its induction of vacuolation (Fig. 9). These results support the involvement of RPTPα in VacA-induced vacuolation in G401 cells.

Identification and functional analysis of the receptors for bacterial toxins is important not only to understand their roles in cell intoxication but also to develop methods to neutralize toxicity following bacterial infection. Our previous studies showed that RPTPβ serves as a receptor for VacA on AZ-521 cells, a human gastric cancer cell line (23, 24); a second protein, p140, was also commonly detected in AZ-521 and AGS, including non-gastric VacA-sensitive cells such as the monkey kidney line COS-7 (19). Here we report that internal amino acid sequence analysis and time of flight-mass analysis of p140, which was isolated from G401 cells, identified their receptor as RPTPα. In agreement with this finding, the binding of V5-tagged RPTPα to VacA was demonstrated by immunoprecipitation using VacA and anti-VacA antibody as shown in Fig. 8. In addition, treatment of G401 cells with RPTPα-morpholino antisense oligonucleotide before exposure to VacA inhibited its induction of vacuolation (Fig. 9). These results support the involvement of RPTPα in VacA-induced vacuolation in G401 cells.

DISCUSSION

Identification and functional analysis of the receptors for bacterial toxins is important not only to understand their roles in cell intoxication but also to develop methods to neutralize toxicity following bacterial infection. Our previous studies showed that RPTPβ serves as a receptor for VacA on AZ-521 cells, a human gastric cancer cell line (23, 24); a second protein, p140, was also commonly detected in AZ-521 and AGS, including non-gastric VacA-sensitive cells such as the monkey kidney line COS-7 (19). Here we report that internal amino acid sequence analysis and time of flight-mass analysis of p140, which was isolated from G401 cells, identified their receptor as RPTPα. In agreement with this finding, the binding of V5-tagged RPTPα to VacA was demonstrated by immunoprecipitation using VacA and anti-VacA antibody as shown in Fig. 8. In addition, treatment of G401 cells with RPTPα-morpholino antisense oligonucleotide before exposure to VacA inhibited its induction of vacuolation (Fig. 9). These results support the involvement of RPTPα in VacA-induced vacuolation in G401 cells.

In transfected COS-7 cells, two forms of overexpressed V5-tagged RPTPα have higher molecular masses (140 and 110 kDa) than the mass calculated (90,559) from RPTPα cDNA (29), suggesting that they have undergone glycosylation. In In transfected COS-7 cells, two forms of overexpressed V5-tagged RPTPα have higher molecular masses (140 and 110 kDa) than the mass calculated (90,559) from RPTPα cDNA (29), suggesting that they have undergone glycosylation. Interestingly, V5-tagged RPTPα of 140 kDa, but not the 110-kDa form, bound VacA, suggesting that the sugar modification of RPTPα is important for binding to VacA. In agreement with this suggestion, neuraminidase treatment diminished the binding to VacA and VacA-induced vacuolation as shown in Figs. 5 and 6.

For many bacterial toxins, interaction with specific cell-surface receptors results in toxin internalization. VacA is known to be internalized by eukaryotic cells (19, 31), and several lines of evidence indicate that VacA-induced cell vacuolation results from VacA activity at an intracellular site (17, 18, 32). Fig. 10 suggests that VacA interaction with RPTPα is required for toxin internalization by G401 cells.

**FIG. 8.** Immunoprecipitation of V5-tagged RPTPα expressed in COS-7 cells with heat-inactivated or native VacA. After transfection of COS-7 cells with vector or V5-tagged RPTPα, proteins in cell lysates were separated by SDS-PAGE in 5% gels, transferred to PVDF membranes, and detected using HRP-conjugated anti-V5 monoclonal antibodies followed by ECL detection (a). To assess VacA binding, lysates were incubated with heat-inactivated (I) or native VacA (A) at 4°C for 1 h before immunoprecipitation with anti-VacA antibody. Immunoprecipitated proteins were separated by SDS-PAGE in 5% gels and transferred to PVDF membranes. RPTPα was identified by its reaction with HRP-conjugated anti-V5 monoclonal antibody (1:2,000) and detected by ECL (b). Positions of molecular mass standards (kDa) and V5-tagged RPTPα are indicated. Data are representative of three separate experiments.

**FIG. 9.** Inhibition of VacA-induced vacuolation in G401 cells by antisense morpholino oligonucleotides. Gene delivery agent EPEI and 1.4 μM RPTPα-morpholino antisense oligonucleotide were incubated in FCS-free DMEM for 20 min at room temperature before addition to G401 cells in 24-well plates followed by incubation for 3 h. After replacement of medium with DMEM containing 10% FCS, cells were incubated for 16 h. Vacuolation of cells caused by incubation with 120 nM VacA for the indicated time was quantified by NRU. As a control (open bar), EPEI was incubated in FCS-free DMEM without antisense oligonucleotide before addition to G401 cells. Data are mean ± S.E. of values from triplicate experiments.

**FIG. 10.** Inhibitory effect of antisense morpholino oligonucleotides on VacA-interlocalization in G401 cells. After G401 cells were transfected with (■) or without (√) the antisense oligonucleotide of RPTPα, cells were incubated with VacA-SS-biotin (120 nM; 10 μg/ml) for the indicated times at 37°C. After treated with MES, internalized VacA labeled with biotin was detected by avidin-HRP. As a control, EPEI was incubated in FCS-free DMEM without antisense oligonucleotide before addition to G401 cells. Data are mean ± S.E. of values from triplicate experiments.
Most RPTPs such as RPTPα and RPTPβ contain two cytoplasmic PTP domains, a membrane proximal domain (D1) and a membrane distal domain (D2), and, in addition, have a single transmembrane segment and an extracellular domain. Structural variability of the extracellular domains of RPTPs suggests selective ligand interactions. The extracellular domain of RPTPβ has recognition sites for many ligands including tenascin, pleiotrophin, contactin, and N-CAM (31). As VacA can bind RPTPα as well as RPTPβ, we compared the sequences of two extracellular regions of significant similarity, which could serve as VacA-binding domains. The amino acid sequence of RPTPα positions 24–74 is 26.7% identical to that of 661–724 in RPTPβ, suggesting that this region of RPTPα may function in VacA binding.

Interestingly, PTPs are a key group of signal transduction enzymes together with protein-tyrosine kinases, and control the levels of cellular protein tyrosine phosphorylation. The enzymes together with protein-tyrosine kinases, and control VacA binding.

Acknowledgments—We thank I. Kato (Medical School of Chiba University) for helpful discussions and K-I, Imagawa (Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan) for analysis of the protein sequence. We thank M. Vaughan (NHLBI, National Institutes of Health, Bethesda, MD) for helpful discussions and critical review of the manuscript.

REFERENCES
1. Del Giudice, G., Covacci, A., Telford, J. L., Montecucco, C., and Rappuoli, R. (2001) Annu. Rev. Immunol. 19, 523–563
2. Peek, R. M., Jr., and Blaser, M. J. (2002) Nature Rev. Cancer 2, 28–37
3. Phadnis, S. H., Iver, D., Janzon, L., Normark, S., and Westblom, T. U. (1994) Infect. Immun. 62, 1557–1565
4. Telford, J. L., Ghiara, P., Dell’Orco, M., Comanducci, M., Burroni, D., Bugnoli, M., Tece, M. F., Censini, S., Covacci, A., Xiang, Z., Papini, E., Montecucco, C., Parente, L., and Rappuoli, R. (1994) J. Exp. Med. 179, 1653–1658
5. Marchetti, M., Arico, B., Burroni, D., Figura, N., Rappuoli, B., and Ghiara, P. (1995) Science 267, 1655–1658
6. Ghiara, P., Marchetti, M., Blaser, M. J., Tummuru, M. K., Cover, T. L., Segal, E. D., Tampkins, L. S., and Rappuoli, R. (1995) Infect. Immun. 63, 4154–4160
7. Eaton, K. A., Cover, T. L., Tummuru, M. K., Blaser, M. J., and Krakowka, S. (1997) Infect. Immun. 65, 3462–3464
8. Supapajarat, V., Ushio, H., Wada, A., Yahiro, K., Okumura, K., Ogawa, H., Hirayama, T., and Ra, C. (2002) J. Immunol. 168, 2603–2607
9. Nakatani, S., Krishnan, E., Oh, S., Yana, R., Marchetti, M., Graham, D. Y., and Genta, R. M. (1997) J. Gastroenterol. 113, 746–754
10. Manetti, R., Massari, P., Burroni, D., de Bernardi, M., Marchini, A., Olivieri, R., Papini, E., Montecucco, C., Rappuoli, R., and Telford, J. L. (1995) Infect. Immun. 63, 4476–4480
11. Leurk, R. D., Johnson, P. T., David, B. C., Kraft, W. G., and Morgan, D. R. (1988) J. Med. Microbiol. 26, 93–99
12. Kimura, M., Goto, S., Wada, A., Yahiro, K., Niidome, T., Hatakeyama, T., Aoyagi, H., Hirayama, T., and Kondo, T. (1999) Microbiol. Pathog. 26, 45–52
13. Galmiche, A., Rasov, J., Deye, A., Cagnol, S., Chambard, J. C., Contamin, S., de Thilivet, V., Just, I., Rici, V., Solcia, E., Van Oberghen, E., and Boquet, P. (2000) EMBO J. 19, 6361–6370
14. Kuck, D., Kolmerer, B., Ikeng-Renert, C., Krammer, P. H., Stremmel, W., and Rudi, J. (2001) Infect. Immun. 69, 5080–5087
15. Czajkowsky, D. M., Iwamoto, H., Cover, T. L., and Shao, Z. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 2001–2006
16. Szabó, I., Brutsche, S., Tembola, F., Moschioni, M., Satin, B., Telford, J. L., Rappuoli, R., Montecucco, C., Papini, E., and Zoratti, M. (1999) EMBO J. 18, 5517–5527
17. Ricci, V., Galmiche, A., Deye, A., Necchi, V., Solcia, E., and Boquet, P. (2000) Mol. Biol. Cell 11, 3897–3909
18. Suzuki, J., Ohno, H., Shibata, H., Wada, A., Hirayama, T., Iiri, T., Ueda, N., Kanamaru, C., Tsuchida, T., Mashima, H., Yasuda, H., and Fujita, T. (2001) J. Clin. Invest. 107, 363–370
19. Garner, J. A., and Cover, T. L. (1996) Infect. Immun. 64, 4197–4203
20. Massari, P., Manetti, R., Burroni, D., Nuti, S., Norais, N., Rappuoli, R., and Telford, J. L. (1998) Infect. Immun. 66, 3981–3984
21. Yahiro, K., Niidome, T., Kimura, M., Hatakeyama, T., Aoyagi, H., Kurazono, H., Imagawa, K., Wada, A., Moss, J., and Hirayama, T. (1999) J. Biol. Chem. 274, 36693–36699
22. Padilla, P. I., Wada, A., Yahiro, K., Kimura, M., Niidome, T., Aoyagi, H., Kumatori, A., Anami, M., Hayashi, T., Fujisawa, J., Saito, H., Moss, J., and Hirayama, T. (2000) J. Biol. Chem. 275, 12200–12206
23. Yahiro, K., Niidome, T., Hatakeyama, T., Aoyagi, H., Kurazono, H., Padilla, P. I., Wada, A., and Hirayama, T. (1997) Biochim. Biophys. Res. Commun. 238, 629–632
24. Shima, D., Estreich, P., Givol, D., and Schlessinger, J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6112–6116
25. Hara, K., Maruki, Y., Long, X., Yoshino, K., Oshiro, N., Hidaya, S., Tokunaga, C., Aurruch, J., and Yonezawa, K. (2002) Cell 110, 177–189
26. Southern, J. A., Young, D. F., Baumgartner, W. K., and Randall, R. E. (1991) J. Gen. Virol. 72, 1551–1557
27. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
28. Qi, M., Ikematsu, S., Maeda, N., Ichihara-Tanaka, K., Sakuma, S., Noda, M., Muramatsu, T., and Kadomatsu, K. (2001) J. Biol. Chem. 276, 15868–15875
29. Kaplan, R., Morse, K., Huebner, K., Corce, C., Avruch, J., and Yonezawa, K. (2002) J. Biol. Chem. 276, 7000–7004
30. Peles, E., Schlessinger, J., and Grumet, M. (1998) Trends Biochem. Sci. 23, 121–124
31. Ricci, V., Sommi, P., Fiecco, R., Romano, M., Solcia, E., and Ventura, U. (1997) J. Pathol. 183, 453–459
32. Molinary, M., Galli, C., Norais, N., Telford, J. L., and Rappuoli, R. (1997) J. Biol. Chem. 272, 25339–25344
