Essential Domain of Receptor Tyrosine Phosphatase β (RPTPβ) for Interaction with Helicobacter pylori Vacuolating Cytotoxin*

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Helicobacter pylori produces a potent exotoxin, VacA, which causes progressive vacuolation as well as gastric injury. Although VacA was able to interact with two receptor-like protein tyrosine phosphatases, RPTPβ and RPTPa, RPTPβ was found to be responsible for gastric damage caused by VacA. To define the region of RPTPβ involved in VacA binding, we made mutants of human cDNA RPTPβ-B, a short receptor form of RPTPβ. Immunoprecipitation experiments to assess VacA binding to RPTPβ-B mutants indicated that five residues (QTTQP) at positions 747–751 of the extracellular domain of RPTPβ-B (which is commonly retained in RPTPβ-A, a long form of RPTPβ) play a crucial role in its interaction with VacA, resulting in vacuolation as well as Git-1 phosphorylation. Transfected cells expressing deletion mutant Δ752, which lacks QTTQP, or the double point mutant Δ747 (T748A,T749A) had diminished vacuolation in response to VacA. Treatment of RPTPβ-B and Δ747 (which have QTTQP at 747–751) with neuraminidase and O-glycosidase diminished their VacA binding, whereas chondroitinase ABC did not have an effect. No inhibitory effect of pleiotrophin, a natural RPTPβ ligand, on VacA binding to RPTPβ-B or Δ747 was observed, supporting the conclusion that the extracellular region of RPTPβ-B responsible for VacA binding is different from that involved in binding pleiotrophin. These data define the region in the RPTPβ extracellular domain critical for VacA binding, in particular the sequence QTTQP at positions 747–751 with crucial threonines at positions 748 and 749 and are consistent with a role for terminal sialic acids possibly because of threonine glycosylation.

Helicobacter pylori is a Gram-negative bacterium, which plays a major role in the development of chronic gastritis and peptic ulcer and is a risk factor for gastric cancer (1–3). Many H. pylori strains isolated from patients contain the cagA gene (cytotoxin-associated gene A) and produce the vacuolating cytotoxin, VacA. Additional H. pylori products, including urease, the neutrophil-activating protein NapA, adhesions, heat-shock protein, and lipopolysaccharide appear to be involved in virulence (1–3). Recent molecular and cellular studies of VacA action have shown that it is a major virulence factor that is involved in the pathogenesis of inflammation in H. pylori-induced gastritis and ulceration (4–6). In mice, orally administered VacA causes degeneration of the gastric mucosa and acute inflammation followed by gastric ulcer disease (7–9). In some eukaryotic cells, VacA induces cytoplasmic vacuolation, leading to death. Purified VacA under denaturing conditions has a molecular mass of ~90 kDa, whereas the native toxin is an oligomer of ~1,000 kDa (10). The VacA protein consists of two functional domains; the 58-kDa C-terminal domain (p58) is responsible for binding to the VacA receptor (11), whereas the 37-kDa N-terminal domain (p37) with 150 amino acids of p58 is cytotoxic when transiently expressed in cultured cells (12, 13).

VacA induces alteration of endosomal function (14, 15), resulting in degenerative vacuolation (16). The phosphorylation of Git1, G protein-coupled receptor kinase-interactor 1, may be responsible for epithelial cell detachment caused by VacA by a mechanism different from that leading to vacuolation (9). Mitochondrial damage (17, 18) and apoptosis (17–20) appear to be vacuolation-independent effects of VacA. Other cytotoxic actions of VacA, such as inhibition of the invariant chain-dependent pathway of antigen presentation by the major histocompatibility complex class II (21), suppression of nuclear translocation of nuclear factor of activated T cells, NFAT, in Jurkat T cells (22, 23), and ATF-2 activation in a gastric cell line (24) have been reported. More recently, Sundrud et al. (25) reported that VacA inhibited the proliferation of primary human CD4+ T cells and that this inhibition was not attributable to VacA effects on NFAT activation or IL-2 expression. Thus, VacA induces multiple effects on epithelial and lymphatic cells, although all of the mechanisms have not been defined.

It is believed that VacA forms anion-selective channels in endosomal membranes through its interaction with lipid (26, 27). Yeast two-hybrid analysis revealed an interaction between VacA and a 54-kDa protein of HeLa cells, consistent with an intracellular target for VacA action (28) and the hypothesis that VacA affects various cellular functions through association with intracellular molecules.

Our previous studies showed that VacA binds on the surface of target cells to two types of receptor-like protein tyrosine
phosphatases (RPTP), RPTPα (29) and RPTPβ (30–32). Indeed, oral administration of VacA to wild-type mice (but not to RPTPβ KO mice) resulted in gastric ulcers, suggesting that RPTPβ is essential for intoxication of gastric tissue by VacA (9). Here we define the extracellular region of RPTPβ, which is required for VacA binding to target cells, leading to vacuolation and Git1 phosphorylation.

MATERIALS AND METHODS

Cells, Culture, and Transient Transfection—COS-7 and BHK-21 (Riken culture bank) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37 °C and 10% CO2. Transfection was performed as described previously (24, 29, 31, 32) with the following modifications. In brief, cells were seeded in 24-well culture plates (0.5 or 1.0 × 10^6 cells in 1 ml of DMEM per well), incubated at 37 °C for 24 h, and rinsed with phosphate-buffered saline (PBS). A mixture of plasmid DNA (1 μg) and 5 μl of FuGENE 6 transfection reagent (Roche Applied Science) in 200 μl of FCS-free DMEM were added to cells. After incubation at 37 °C for 3 h, 1 ml of DMEM was added; the cells were then incubated at 37 °C for 24 h before analysis.

Purification of VacA—VacA was purified from H. pylori ATCC49503 strain culture supernatant using an anti-VacA antibody column as described (24, 29). VacA was precipitated from culture supernatant with 50% saturated ammonium sulfate. Precipitated proteins were dialyzed against RX buffer (10 mM KCl, 0.3 mM NaCl, 0.35 mM MgCl2, and 0.125 mM EGTA in 10 mM HEPES, pH 7.3) and applied to an anti-VacA-specific IgG antibody column (10 mg of IgG/2 mg dried resin) equilibrated with RX buffer. After washing the column with RX buffer, VacA was eluted in 50 mM glycine-HCl buffer, pH 1.0, which was immediately neutralized with 1 M Tris-HCl, pH 10. After gel filtration on Superose 6HR 10/30 equilibrated with RX buffer, VacA was concentrated (30). DNA Preparation and Standard Reverse Transcriptase-PCR—Total RNA (0.5 μg) was extracted from AZ-521 cells by using ISOSGEN (Nippon Gene, Tokyo, Japan) and reverse transcribed into single-strand cDNA (1st strand cDNA synthesis kit, Roche Applied Science) using random primers. The resulting cDNA (0.25 μg) was used as a template for PCR to amplify specific cDNA regions of human RPTP-A (long form of human RPTPβ) and RPTPβ-B (short form of human RPTPβ) by PCR using primers RPTPβ-Afwd, RPTPβ-Bfwd, and RPTPβ-rev (see Table I). After initial denaturation for 1 min at 95 °C, 35 cycles of denaturation (1 min, 94 °C), annealing (2 min, 55 °C), and elongation (2 min, 72 °C) were followed by a final elongation for 7 min at 72 °C. For control amplifications of the glyceraldehyde-3-phosphate dehydrogenase mRNA, similar cycling conditions were used.

The abbreviations used were: RPTP, receptor-like protein-tyrosine phosphatase; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; PTN, pleiotrophin; HA, hemagglutinin; PVDF, polyvinylidene fluoride; PTN, pleiotrophin.
Construction of RPTPβ-B and Its Deletion Mutants—To construct the RPTPβ-B gene, first strand cdna described above was amplified using primers, RPTPβ-fwd and RPTPβ-rev. Sequence analysis of the amplified fragment confirmed its identity with human RPTPβ-B (34). RPTPβ-A was cloned into the pBK-CMV vector (32, 35). The PCR products cut with BamHI and ClaI were inserted into the BamHI and ClaI site of pBK-CMV-RPTPβ-A. This resulting pBK-CMV-B-WT-RPTPβ cDNA encoded a sequence identical to that of RPTPβ-B as determined by Levy et al. (34). However, we could not succeed in expressing RPTPβ-B in transfected cells using pBK-CMV-B-WT-RPTPβ. Therefore we prepared HA-tagged pEF-ROS-RPTPβ-B; primers used for the RPTPβ signal peptide region were RPTPβ-B1s (GCAGCC was added to the primer to create an NheI site) and RPTPβ-B1a (GGCTGCA was added to the primer to create a PstI site) (see Table I). The PCR products were first subcloned into the NheI and PstI sites of the pBK-CMV vector. Next, HA oligonucleotides HA-a (sense) and HA-a (antisense) (TCCGAC and CTGCAG were added to the primer to create a site for Sall and PstI) were annealed and inserted into multicloning sites of PstI and Sall on the pBK-CMV vector, which contained a sequence encoding a signal peptide region; these constructs were then digested with NheI and KpnI. These fragments were treated with Klenow and then ligated into the pEF-BOS vector using XbaI sites. RPTPβ-B was amplified using pBK-CMV-B-WT-RPTPβ DNA as a template and primers, RPTPβ-B2s (ACTAGT was added to the primer to create an HA site) and RPTPβ-rev, and the resulting cDNA was subcloned into a pBlueScript vector, which was then digested with ClaI and SmaI. These fragments were cloned into SpeI and Clai sites of the pEF-BOS vector, into which the signal peptide region and HA tag had been inserted. The primers listed on Table I were used to construct various deletion and point mutants of HA-B-WT. These PCR products were first subcloned into the pCR2.1-TOPO vector (Invitrogen), which was then digested with SpeI and Clai. These fragments were cloned into the SpeI and Clai sites of the pEF-BOS-HA-B-WT vector.

**Cell Lysis, Immunoprecipitation, and Blotting**—Cells were washed once with cold PBS and harvested in TNE buffer (40 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA), followed by centrifugation at 5,000 × g for 2 min. Cells were suspended and lysed in 500 μl of T-lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 10% glycerol, 100 mM NaCl, 5 μg/ml leupeptin) for 20 min on ice. After centrifugation (17,000 × g, 20 min) to remove insoluble material, the supernatants (60 μg, 250 μl) were incubated with 30 μl heat-inactivated VacA or VacA for 1 h and then incubated overnight with anti-VacA IgG. Protein A-Sepharose CL-4B beads (25 μl of 50% bead suspension solution per sample) (Amersham Biosciences) were added, followed by incubation for 1 h. Beads were washed three times with T-lysis buffer and boiled in SDS-sample buffer before separation of precipitated proteins by SDS-PAGE. Proteins were transferred to PVDF membranes, reacted with specific antibodies using standard procedures, and visualized using enhanced chemiluminescence (ECL, Amersham Biosciences). To immunoprecipitate wild-type or deletion mutants of RPTPβ containing an HA tag, which were expressed in the COS-7 cells, anti-HA antibody (instead of anti-VacA antibody) was added to the supernatants of cell lysates. An anti-human RPTPβ mouse monoclonal antibody (Transduction Laboratories), which had been prepared by immunization with the 2098–2207 region of human RPTPβ, was used for detection of RPTPβ-A and RPTPβ-B expressed in AZ-521 cell after immunoprecipitation and Western blotting analysis.

**Git1 Phosphorylation**—BH-21 cells (2 × 10⁶), which lack RPTPβ but contain its substrate Git1, were cultured on Matrigel-coated plates for 24 h in DMEM containing 10% FCS and then transfected with RPTPβ mutant expression plasmids (9). After cultivation for 24 h, cells were washed twice with ice-cold PBS; this was followed by treatment with N-lysine buffer (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.2% deoxycholic acid, 1 mM sodium orthovanadate, 1 mM NaF, and protease inhibitor) for 30 min on ice. After centrifugation (15,000 × g, 20 min), 1 μl of anti-Git1 antibody (Transduction Laboratories) and 25 μl of Protein A-Sepharose CL-4B (Amersham Biosciences) were added to the supernatants (320 μg, 400 μl); this was followed by incubation for 3 h at 4 °C. Beads were washed three times with N-lysine buffer and boiled in SDS-sample buffer before separation of precipitated proteins by SDS-PAGE. Proteins were transferred to PVDF membranes, reacted with anti-phosphotyrosine monoclonal antibody-HRP (1: 1000, Amersham Biosciences) or anti-Git1 monoclonal antibody (1: 2000, Transduction Laboratories) and visualized using ECL. Densitometric analysis was performed to compare the intensities of the visualized bands.

**Neuraminidase and O-Glycosidase Treatments**—After transfection of wild-type RPTPβ or its deletion mutant cDNA constructs into COS-7 cells and growth for 24 h, the cells (3 × 10⁹) were lysed with T-lysis buffer, which was followed by centrifugation (20 min, 17,000 × g). Samples (6 μg of supernatant) (20 μl) were added to a mixture of 20 μl of phosphate buffer, pH 7.2, 5 μl of 10% Nonidet P-40, and 1 μl of neuraminidase (Roche Applied Science, 10 milliunits/μl) or O-glycosidase (Roche Applied Science, 0.5 or 1 milliunits/μl); this was followed by incubation at 37 °C for 3 or 24 h for neuraminidase or O-glycosidase treatment, respectively. Immunoprecipitation was performed with anti-VacA antibody after addition of VacA or a heat-inactivated VacA. Immunoprecipitated proteins were separated by SDS-PAGE in 7.5% gels and transferred to PVDF membranes for analysis.

**RESULTS**

Mapping of Responsible Region of RPTPβ-B to Bind VacA—Four isoforms of RPTPβ are known; RPTPβ-A is the long receptor form. RPTPβ-B is the short form lacking a serine glycine-rich region in the extracellular domain, and two soluble forms, phosphacan and phosphacan short form, lack transmembrane and intracellular domains (36). Among four isoforms, RPTPβ-A and RPTPβ-B, which contain intracellular domains (Fig. 1A), may function in signal transduction events...
Fig. 2. Immunoprecipitation of RPTPβ-B (B-WT) and its mutants transiently expressed in COS-7 cells. A, diagram of HA-tagged B-WT and its mutants with deletion and amino acid replacements. CAH, carbonic anhydrase; FN, fibronectin type III; DI, DII, two cytoplasmic protein tyrosine phosphatase domains. B, lysates of cells transfected with B-WT or its mutant constructs were mixed with heat-inactivated (IA) or untreated 30 nM VacA (A) and immunoprecipitated with anti-VacA IgG. Precipitated proteins were separated by SDS-PAGE, transferred to PVDF
involved in VacA toxicity. Levels of RPTPβ-A and RPTPβ-B mRNA in AZ-521 cells were determined by reverse transcriptase-PCR analysis using primers RPTPβ-Afwd, RPTPβ-Bfwd, and RPTPβ-rev, respectively (Table I). Gastric cell line AZ-521 expressed both RPTPβ-A and RPTPβ-B at the mRNA (Fig. 1B) or protein (Fig. 1C) level. To determine the region of RPTPβ that is required for VacA binding, we used RPTPβ-B cDNA to prepare several mutant expression constructs (nine deletion mutants and three mutants with amino acid substitutions) with a HA-tag sequence in a pEF-BOS vector (Fig. 2A). VacA interaction with HA-tagged RPTPβ-B mutants, which were transiently expressed in COS-7 cells, was tested by immunoprecipitation using anti-VacA antibody. Among nine deletion mutants tested, VacA interacted with all except Δ747 (Fig. 2B). The difference between these eight deletion mutants and Δ752 in VacA binding was not caused by differences in amounts of mutant proteins, which were not significantly different in the nine populations of transfected cells, as assessed by Western blot analysis using anti-HA antibody (Fig. 2C).

Significant Role for VacA Interaction of Terminal Sialic Acids, O-Glycosylation Site, and Threonine at Positions 748 and 749 in RPTPβ-B—Two RPTPs that bind VacA (9, 29, 31, 32) are glycoproteins. As the terminal sialic acid of RPTPα in G-401 cells might be required for VacA binding (29), we examined whether neuraminidase or O-glycosidase treatment of RPTPβ expressed in COS-7 cells might decrease VacA binding (Fig. 3). After incubation with neuraminidase or O-glycosidase, the lysates of COS-7 cells expressing wild-type RPTPβ-B (B-WT) or Δ747 were incubated with VacA or heat-inactivated VacA, which was followed by immunoprecipitation with anti-VacA antibodies. VacA binding to the cell lysate of COS-7 cells expressing B-WT was reduced by neuraminidase treatment as shown in Fig. 3A. Neuraminidase or O-glycosidase treatment diminished VacA binding to Δ747, suggesting that terminal sialic acids and O-linked glycosylation of RPTPβ-B also played a pivotal role in VacA binding (Fig. 3, B and C).

Because RPTPβ-B contains an in-frame deletion of amino acids 755–1613 of RPTPβ-A, both RPTPβ-A and RPTPβ-B retain QTTQP at positions 747–751 of the extracellular domains. We investigated whether the QTTPQ sequence at 747–751 with two potential O-glycosylation sites may play an important role in interaction with VacA. Taking into account the potential significance of terminal sialic acids of RPTPβ, threonine was replaced with alanine at putative O-glycosylation sites as described in the report on O-glycosylation consensus sequence (37). As proline at +3 and/or –1 positions strongly favors glycosylation, two threonines at positions 748 and 749 in RPTPβ-B sequence were assumed to be O-glycosylated. To obtain further insights into the function of QTTPQ at 747–751 of RPTPβ-B for VacA binding, we generated three mutants, Δ747(T748A), Δ747(T749A), and Δ747(T748A,T749A) and then analyzed their VacA-binding abilities by immunoprecipitation (Fig. 4). Two mutants, Δ747(T748A) and Δ747(T749A), with single amino acid replacements bound to VacA to an extent similar to that of Δ747 (Fig. 4A), but the double mutant Δ747(T748A,T749A) did not bind VacA (Fig. 4B). All RPTPβ point mutants were present at similar levels in transfected cells based on Western blotting with anti-HA antibody. These results indicate that the two threonines at positions of 748 and 749 play an important role in VacA binding.

VacA-induced Vacuolation and Git1 Phosphorylation in Transfected Cells Expressing B-WT and Its Mutants—When cells expressing B-WT, two deletion mutants, and three point mutants were exposed to 120 nm VacA in the presence of 5 mM NH₄Cl for 8 h, vacuolation appeared in cells expressing Δ747, Δ747(T748A), or Δ747(T749A) at levels similar to that in cells expressing B-WT (Fig. 5A). However, cells expressing Δ747(T748A,T749A) or Δ752 or cells transfected with vector alone showed decreased vacuolation. We further examined whether VacA-induced phosphorylation of Git1 in BHK-21 cells transfected with Δ747, Δ747(T748A,T749A) or vector. As shown in Fig. 5, B and C, VacA induced phosphorylation of Git1 membranes, and detected with anti-RPTPβ antibody. C, proteins immunoprecipitated from lysates of COS-7 cells transfected with vector, B-WT, or its mutant constructs with anti-HA antibodies were separated, and blots were reacted with anti-RPTPβ antibodies. Arrows indicate immunoprecipitated B-WT and its mutants, respectively. Data are representative of three separate experiments.
in cells expressing Δ747 but not Δ747(T748A,T749A), suggesting that two threonines at positions of 748 and 749 play an important role in VacA-induced Git1 phosphorylation.

**DISCUSSION**

Numerous studies have shown that RPTPβ, a chondroitin sulfate proteoglycan with an extracellular region containing a carbonic anhydrase-like domain and a single FNIII domain, plays an important role in cell migration (38), differentiation (39), synaptogenesis (40), synaptic function (41), and myelination (42, 43) in the central nervous system. Recently, we established that RPTPβ functions as a receptor for VacA (24, 29, 31, 32) and that RPTPβ, expressed in gastric tissue, is responsible for gastric injury caused by VacA (9). Notably, as there was no difference in vacuole development between wild-type mice and RPTPβ-deficient mice, we concluded that RPTPβ is not essential either for the internalization of VacA or for vacuolation in gastric epithelial cells. However, we subsequently showed that VacA bound to RPTPβ, which is ubiquitously expressed in many tissues including stomach (44), suggesting the presence of an alternative receptor for VacA in RPTPβ-deficient mice. In agreement with the significance of toxin internalization for...
vacuolation through its RPTPβ binding (29), we found that RPTPβ functions in VacA-induced vacuolation (31) and inter-
nalization (data not shown). Taken together with the fact that
PTN induced severe gastritis in wild-type mice but not in
RPTPβ-deficient mice without cellular vacuolation (9), it ap-
pears that VacA induces gastric ulcers through RPTPβ-dep-
endent signal transduction events not leading to cellular
vacuolation.

To identify the essential structure of RPTPβ required for
VacA binding, we examined whether various mutants of
RPTPβ-B, a short form of RPTPβ, interact with VacA by im-
munoprecipitation and functional analyses (Fig. 1). Three
RPTPβ-B mutants, Δ747, Δ747(T748A), and Δ747(T749A) bound to VacA, whereas two mutants, Δ752 and Δ747(T748A,
T749A) did not (Figs. 2 and 4). Moreover, treatment of cells
expressing B-WT or Δ747 with neuraminidase or O-glycosidase
diminished VacA binding (Fig. 3), suggesting that glycosylation
in the extracellular region at positions 747–751 is important for
VacA binding. In addition, PTN, which is an 18-kDa heparin-
binding growth factor and a natural ligand for RPTPβ (45, 46),
did not competitively interfere with VacA binding to B-WT and
Δ747 (Fig. 6), indicating that the RPTPβ binding site for VacA
is different from that for PTN. Of note, PTN binds chondroitin-
sulfate, which is removed by chondroitinase ABC, resulting in
a significant reduction in PTN binding to RPTPβ-B (38, 47).

This result is consistent with our finding that treatment of cells
expressing B-WT and Δ747 with heparitinase or chondroiti-
nase ABC did not affect their molecular mass and VacA binding
(data not shown).

Our findings indicate that the common QTTQP sequence at
747–751 in RPTPα-A and RPTPβ-B is required for VacA binding. O-Glycosylation of two threonines at position 748 and 749 and the presence of a terminal sialic acid promoted VacA binding and vacuolating activity (Fig. 5A). Fig. 5B also shows that two threonines at position 748 and 749 in RPTPβ-A and RPTPβ-B were required for VacA-induced Git1 phosphorylation (9).

Signaling functions of RPTPα and RPTPβ have been predominantly studied primarily in mammalian cell systems, which has led to important insights into regulation of RPTPα activity as well as its potential ligands and substrates. The major action of RPTPα is as a positive regulator of Src and Src family kinases through dephosphorylation (48). RPTPβ promotes cell adhesion, neurite growth, and migration (49). Thus, both RPTPα and RPTPβ have the potential to regulate signal transduction through tightly controlled dephosphorylation of tyrosyl residues in proteins by the binding of specific extracellular ligands or cytoplasmic proteins. A variety of extracellular ligands of RPTPα have been identified; PTN, N-CAM, Ng-CAM, tenascin, contactin, and midkine (49). Among these RPTPα ligands, PTN and midkine are secreted proteins that exert their effects through inhibition of RPTPβ phosphatase activity. Most known ligands of RPTPα seem to be extracellular but cytoplasmic (e.g., GRB2, which binds the Tyr-789 (mouse) or Tyr-798 (human) of RPTPα) (50, 51). As the extracellular domain of RPTPα is small, it is conceivable that its function may be to tether tyrosine phosphatase activity to the plasma membrane (49). VacA is the first extracellular ligand to be discovered for RPTPα. As the potential roles of VacA as a ligand for RPTPα and RPTPβ are only poorly understood, further studies are needed to determine the implications of VacA-receptor complexes for intracellular signaling.

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