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Cytotoxicity and recognition of receptor-like protein tyrosine phosphatases, RPTPα and RPTPβ, by *Helicobacter pylori* m2VacA

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Summary

Helicobacter pylori **vacuolating cytotoxin, VacA, induces vacuolation in mammalian cell lines. Sequence differences in the middle of VacA molecules define two families, termed m1VacA and m2VacA, which differ in cell specificity. Similar to m1VacA, m2VacA is activated by acid or alkali, which enhances its binding to cells. Immunoprecipitation experiments showed that, in AZ-521 cells, activated m2VacA, similar to m1VacA, binds to two receptor-like protein tyrosine phosphatases, RPTPα and RPTPβ suggesting that activated m2VacA as well as m1VacA may contribute to gastrointestinal disease following *H. pylori* infection. G401 cells express RPTPα, not RPTPβ, and responded to both m1VacA and m2VacA. HeLa cells likewise expressed RPTPα, not RPTPβ, but, in contrast to other cell lines, responded poorly to m2VacA. m1VacA associated with RPTPα of HeLa cells to an extent similar to that in other toxin-sensitive cells, whereas activated m2VacA bound HeLa cell RPTPα less well, consistent with its low vacuolating activity against these cells. The molecular mass of RPTPα from HeLa cells is less than that of the protein from G401 cells, although their extracellular amino acid sequences are virtually identical, with only two amino acid differences noted. Different post-translational modifications of RPTPα in HeLa cells may be responsible for the reduced susceptibility to m2VacA.**

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Introduction

It is now widely accepted that *Helicobacter pylori* plays a central role in the pathogenesis of gastritis, peptic ulcer disease and gastric adenocarcinoma (Warren and Marshall, 1983, Parsonnet *et al*., 1991; Axon, 1999). Among the virulence factors elaborated by *H. pylori*, vacuolating cytotoxin, VacA, has been shown to cause progressive vacuolation and death of epithelial cells (Del Giudice *et al*., 2001). VacA under denaturating conditions has a molecular mass of 87–95 kDa, whereas the native toxin is an oligomeric complex of about 1000 kDa (Manetti *et al*., 1995). Genotyping of clinical isolates revealed two regions of the *vacA* gene where sequence differences identify *H. pylori* families. Two families (s1 and s2) are differentiated by sequences at the 5′ end of the *vacA* gene. Two additional families (m1 and m2) are distinguished based on mid-regions of the *vacA* gene (Atherton *et al*., 1995). s1/m1 and s1/m2 types of VacA predominated in clinical isolates of *H. pylori*, whereas s2/m1 and s2/m2 occurred quite rarely (Papini *et al*., 2001). A 12 amino acid hydrophilic amino-terminal segment, present in the s2 type but absent from s1, diminished the cytotoxic effects of VacA (McClain *et al*., 2001). Strains encoding s1/m1 *vacA* gene typically produce m1VacA with cytotoxic activity on human cervical carcinoma HeLa cells, whereas m2VacA, produced by strains with the s1/m2 vacA gene, induced vacuoles in primary cultured human gastric cell lines as well as non-gastric epithelial RK13 cells, but not in HeLa cells (Pagliaccia *et al*., 1998). As s1/m2 strains

produce low levels of toxin, it is likely that m1VacA is responsible for more epithelial damage than m2VacA (Atherton *et al*., 1997). To the contrary, however, Go *et al*. (1998) reported that m2VacA is associated with duodenal ulcer, suggesting that *H. pylori* strains producing either m1VacA and m2VacA were associated with gastric pathology.

We reported that m1VacA interacts with target cells by binding to two types of receptor-like protein tyrosine phosphatases (RPTPs), i.e. RPTPα and RPTPβ, resulting in toxin internalization and vacuolation of the human gastric adenocarcinoma cell lines AZ-521 and G401 (Yahiro *et al*., 1999; Padilla *et al*., 2000; Yahiro *et al*., 2003). Furthermore, oral administration of m1VacA to wild-type mice, but not RPTPβ KO mice, resulted in gastric ulcers, suggesting that RPTPβ is essential for intoxication by m1VacA in gastric tissue (Fujikawa *et al*., 2003). Our recent studies showed that glycosylation in the extracellular region of two RPTPs is important for m1VacA binding (Yahiro *et al*., 2003; 2004). Here we report that exposure of m2VacA to acid or alkali markedly enhanced toxin binding to both RPTPα and RPTPβ, resulting in enhancement of vacuole formation in AZ-521 and G401 cells. HeLa cells, which expressed an RPTPα, smaller than that from other cells, responded poorly to m2VacA.

Results and discussion

Purification of m2 type of vacuolating cytotoxin

Of the five *H. pylori* strains that produce VacA with an m2 mid-region, OK210 yielded the most protein, which reacted with anti-VacA IgG and its molecular weight of 95 kDa was slightly higher than those of m1VacA and m2VacA from strains OK 139, OK 160 and OK 204 on sodium dodecyl sulphate (SDS)-polyacrylamide gels electrophoresis. Sequencing of genes from strains OK210 and OK160 revealed the presence of 3969 bp and 3909 bp ORFs, encoding proteins of 1323 and 1303 amino acids respectively. The s1 signal peptide and the m2 mid-region are consistent with a molecular mass of m2VacA from OK210 similar to that of a 94 kDa s2/m2 VacA from *H. pylori* strain Tx30a (Atherton *et al*., 1995). Homology of total amino acid residues of m2VacA from OK210 to those of m1VacA from strain 60190 was found to be 83.8%. As the m2VacA of OK210 contained the same number of amino acids (92.4% mean amino acid identity) reported in s1m2VacA from strain 95–54, which lacked toxicity against HeLa cells (Pagliaccia *et al*., 1998), and the largest amount of m2VacA was produced in OK210, we purified m2VacA from OK210 by precipitation with 50% saturated ammonium sulphate followed by hydroxyapatite and Superose 6 chromatography. A 95 kDa protein was detected as a single band by SDS-PAGE in 7.5% gels stained with Coomassie brilliant blue (Fig. 1A). All three samples also reacted with an anti-m1VacA IgG as shown by Western blotting (Fig. 1B). It is well known that the 88 kDa purified m1VacA commonly undergoes degradation, yielding 33- and 55 kDa fragments (Nguyen *et al*., 2001; Torres *et al*., 2004). Likewise, m2VacA may undergo degradation. Two fragments visible as weak bands (at a position of about 55 kDa and at the front of an SDS 7.5% gel) reacted with anti-m1VacA antibody on Western Blotting.

Vacuolating activities of m1VacA and m2VacA on gastric and non-gastric cell lines

The m1VacA used here was activated after VacA affinity chromatography and induced vacuolation in gastric AZ-521 cells and non-gastric HeLa, RK13 and G401 cells

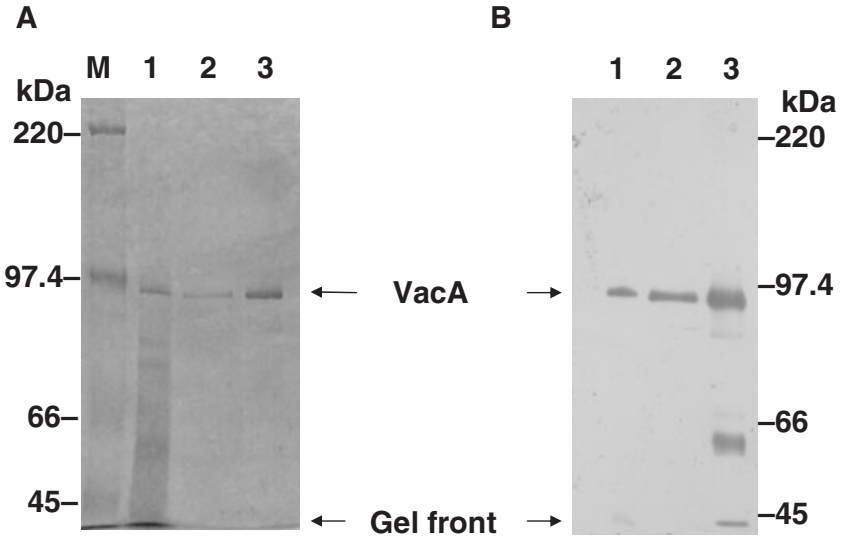

Fig. 1. Purification of m2VacA from strain OK210. Purity of m2VacA from *H. pylori* OK210 in all stages of purification was analysed by SDS-PAGE (A) and Western Blotting (B).
Lane 1, 50% ammonium sulphate precipitation (1 μg protein) of *H. pylori* OK120 culture supernatant.
Lane 2, partially purified m2VacA (1 μg protein) from hydroxyapatite column chromatography.
Lane 3, purified m2VacA (1 μg protein) from Superose 6 HR filtration chromatography.
Lane M, molecular mass standards (kDa) separated by SDS-PAGE in 7.5% gel.
CBB stain (A) and immunoblot (B) showed a 94 kDa band in all stages of purification. Positions of molecular mass standards (kDa) and m2VacA from strain OK120 are indicated. Data are representative of three separate experiments.

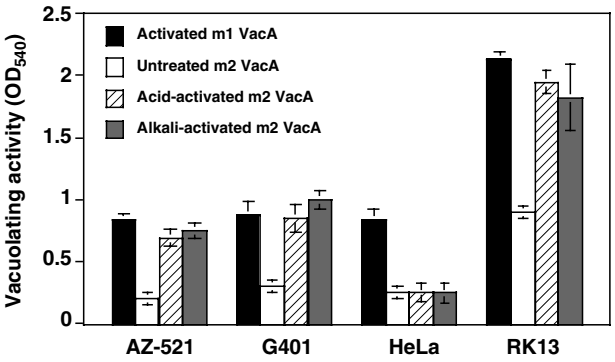

Fig. 2. Acid- and alkali-activation of m2VacA. Vacuolating activities of m1VacA and m2VacA on AZ-521, G401, HeLa and RK13 cells. Four types of cells, AZ-521, G401, HeLa and RK13 were incubated with activated m1VacA, untreated m2vacA, and acid- and alkali-activated m2VacA at 120 nM for 8 h before vacuolation was quantified by NRU assay. Data are means ± SEM of values from three experiments.

(Fig. 2). On the other hand, native untreated m2VacA showed significant vacuolating activity with RK13 cells, but was only weakly active with AZ-521, G401 and HeLa cells. To determine whether acid- or alkali-treated m2VacA would increase vacuole formation as did acid- and alkali-activated m1VacA (de Bernard *et al*., 1995; Yahiro *et al*., 1999), vacuolation in AZ-521, G401, HeLa and RK13 cells caused by m2VacA that had been exposed to pH 1.5 or 11.5 was quantified by NRU (Neutral Red Dye Uptake) assay. Acid- or alkali-treated m2VacA had greater vacuolating activity in AZ-521 and G401 cells, but not in HeLa cells, than did untreated m2VacA. Activated m1VacA and m2VacA induced vacuolation in AZ-521 cells with similar dependence on concentration (data not shown).

Interaction of m2VacA with RPTPα and RPTPβ in AZ-521 cells was increased by acid or alkali-treatment

We further examined receptor recognition of m2VacA using AZ-521 cells. As shown in Fig. 3, RPTPβ was immunoprecipitated from AZ-521 cells with anti-m1VacA IgG and protein A-Sepharose CL-4B from lysates of cells that had been incubated with activated, but not with heat-inactivated toxin. In agreement with limited vacuole formation in AZ-521 cells treated with m2VacA, native m2 VacA bound very weakly to p250 (Fig. 3A), which was confirmed by the immunoblot using anti-RPTPβ for detection

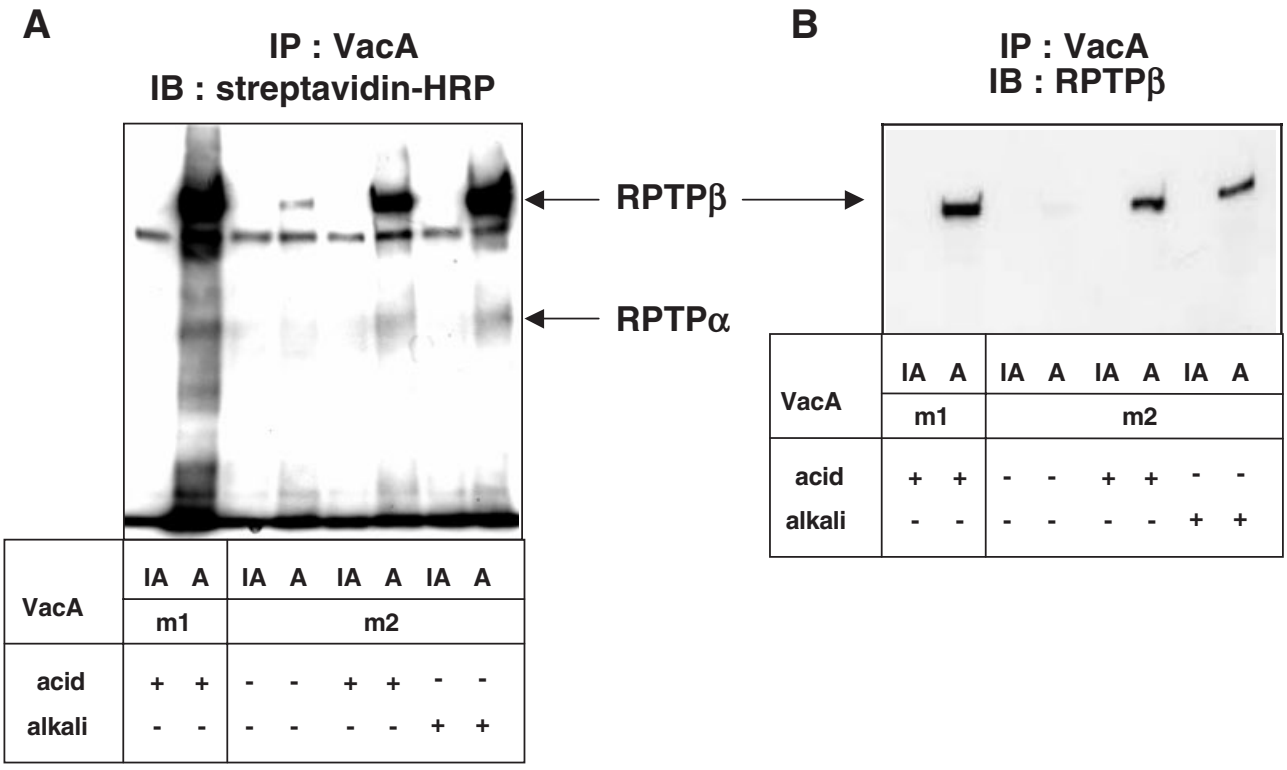

Fig. 3. Immunoprecipitation of m2VacA binding proteins in AZ-521 cells.
A. Cell surface proteins of AZ-521 were biotinylated, and active (A) or inactive (IA) m2VacA was added to the cell lysates before immunoprecipitation (IP) of VacA-binding proteins with anti-VacA antibody. Antibody-bound proteins were collected by addition of protein A Sepharose CL-4B, separated by SDS-PAGE in 5% gels, and transferred to PVDF membranes, which were incubated with HRP-conjugated streptavidin, followed by ECL detection. Positions of molecular mass standards (kDa) are on the left.
B. RPTPβ was detected with anti-RPTPβ antibody. Data are representative of three separate experiments.

(Fig. 3B), suggesting association of m2VacA with RPTPβ; the presence of RPTPβ is responsible for gastric injury induced by VacA in mice (Fujikawa *et al.*, 2003; Peek, 2003). Activated m2VacA, however, bound and immunoprecipitated RPTPβ to almost the same extent as did acid-activated m1VacA. It is clear from Fig. 3A that activated m2VacA also bound RPTPα in AZ-521 cells.

Strong association of acid-activated m2VacA with RPTPα in G401 cells and its weak interaction with RPTPα in HeLa cells

To assess the interaction of acid-activated m2VacA with RPTPα, we examined the effect of acid treatment of m2VacA on its binding to RPTPα in G401 and HeLa cells, which lack RPTPβ. Although acid-activated m1VacA and acid-activated m2VacA bound to G401 cells at similar levels, as assessed by indirect immunofluorescence and flow cytometry, activated m2VacA binding to HeLa cells was very low (Fig. 4). In agreement with these results, activated m1VacA and m2VacA associated with RPTPα of G401 cells at similar levels (Fig. 5A and B) as assessed by immunoprecipitation using anti-RPTPα antibody, whereas binding of activated m2VacA to RPTPα of HeLa cells was significantly less than that of activated m1VacA (Fig. 5C and D).

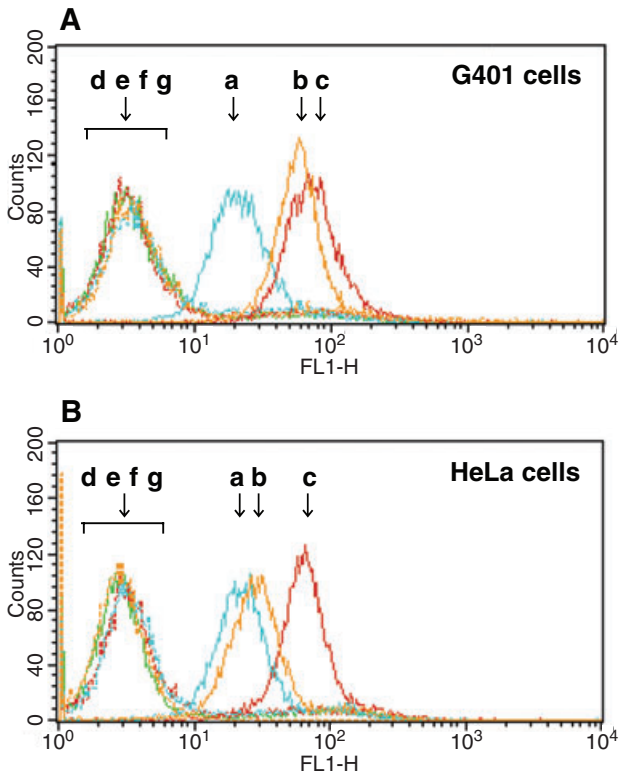

Fig. 4. FACScan analysis of activated m1VacA and m2VacA binding to G401 and HeLa cells. Suspensions of G401 (A) or HeLa cells (B) were incubated with 120 nM native m2VacA (a) or acid-activated m2VacA (b) at 4°C for 30 min. As a control, cells were incubated with activated m1VacA (c), heat-inactivated native m2VacA (d), heat-inactivated, acid-activated m2VacA (e), heat-inactivated m1VacA (f) and without toxin (g). Toxin binding was quantified by flow cytometry using anti-m1VacA IgG and Alexa Fluor ^{TM488}-labelled goat anti-rabbit IgG. Data are representative of three separate experiments.

Differences in molecular mass of RPTPα

To clarify the discrepancy of RPTPα binding between G401 and HeLa cells, we compared the molecular masses of RPTPα (Fig. 6). By Western blot analysis using anti-RPTPα antibody, the mobility of RPTPα from HeLa cell lysates on SDS-PAGE was greater than that from G401 cell lysate, which was similar to that of RPTPα from AZ-521 and RK13 cells, indicating the molecular mass of RPTPα in HeLa cells was smaller than that of RPTPα from other toxin-sensitive cells (Fig. 6A). In agreement with these data, the mobility of RPTPα from lysate of V5-tagged RPTPα-transfected HeLa cells on SDS-PAGE was faster than that of V5-tagged RPTPα synthesized in G401 cells (Fig. 6B). The difference in size was not a result of the difference in RPTPα sequences of G401 and HeLa cells, which were found to be virtually identical to the published RPTPα sequence of the RPTPα isoform (Daum *et al.*, 1994). Glycine was present at position 66 of HeLa RPTPα in place of serine and isoleucine at position 99 in place of valine when compared with G401 cell RPTPα (data not shown).

Differences in molecular mass of RPTPα from G401 and HeLa cells after N*-glycosidase F and neuraminidase treatments*

After incubation with *N*-glycosidase F and neuraminidase, proteins in lysates of G401 and HeLa cells were analysed by Western blotting with anti-RPTPα antibodies. Consistent with our previous report (Yahiro *et al.*, 2003), treatment of G401 cells with *N*-glycosidase F resulted in appearance of a smaller RPTPα of about 135 kDa; neuraminidase treatment did not change the apparent mobility of RPTPα (Fig. 7A). Molecular size of HeLa RPTPα was not changed by either enzyme treatment. The migration of HeLa RPTPα on SDS-PAGE in 5% gels was found to be almost the same as that of *N*-glycosidase-treated G401 RPTPα from G401 cells. However, this small RPTPα from G401 cells bound m1VacA as well as m2VacA (Fig. 7B). Thus, N-glycosylation of G401 cell RPTPα appears not to be relevant for binding of VacA.

The 123-amino acid extracellular domain of RPTPα is clearly a potential site of specific interactions with ligands (Johnson and van Vactor, 2003). Moreover, we showed that extensive glycosylation could also play an important role in m1VacA interaction of RPTPα (Yahiro *et al.*, 2003)

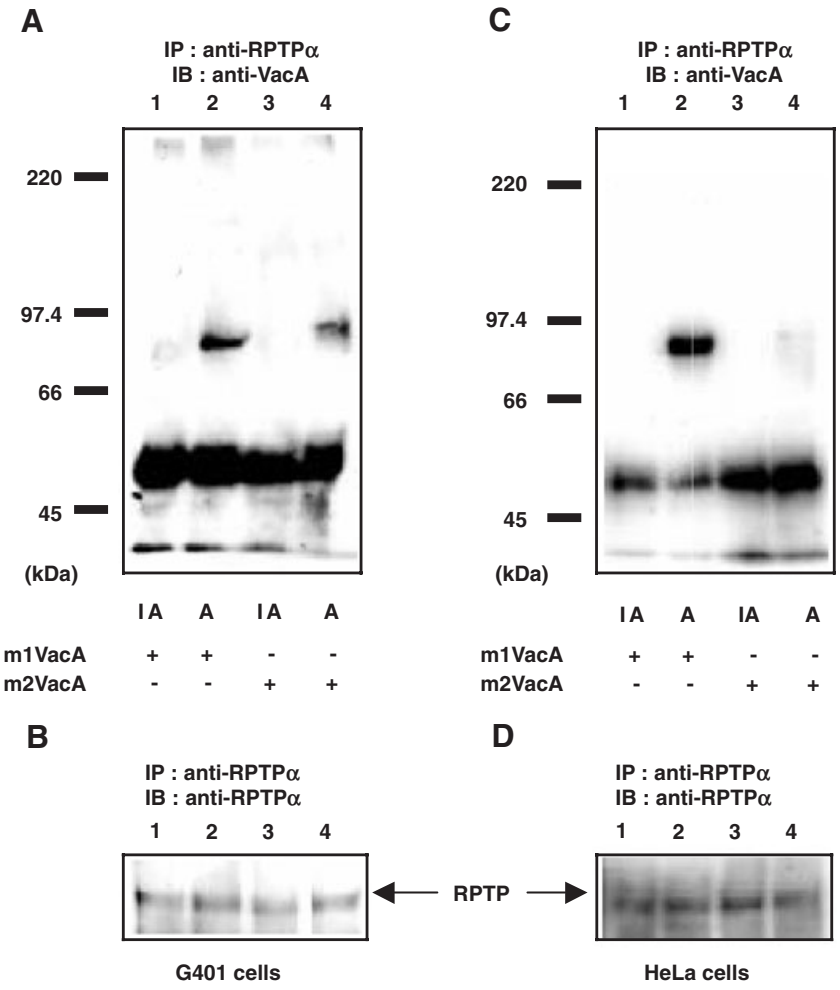

Fig. 5. Specific binding of activated m1VacA and m2VacA to RPTPα of G401 and HeLa cells. Proteins solubilized from G401 (A and B) or HeLa cells (C and D) were incubated with heat-inactivated (IA) or activated (A) m1VacA and m2VacA before immunoprecipitation with anti-RPTPα antibody. Following addition of protein A-Sepharose CL-4B, immunoprecipitated proteins were separated by SDS-PAGE in 5% gels and transferred to PVDF membranes. VacA (A and C) was detected by incubation with anti-VacA antibody (IB) and HRP-conjugated anti-rabbit immunoglobulins, followed by ECL detection. To detect RPTPα in immunoprecipitated samples from G401 (B) and HeLa cells (D), membranes were incubated with anti-RPTPα antibody (IB) and detected by ECL after addition of HRP-conjugated anti-rabbit immunoglobulins. Data are representative of three separate experiments.

as in m1VacA interaction of RPTPβ (Yahiro *et al*., 2004). It should be noted that the *O*-glycosylation of RPTPα may be required for VacA binding because treatment of G401 cells with *O*-glycosidase diminished the binding of m1VacA as well as m2VacA to RP′TPα (data not shown). The binding of m2VacA to RPTPβ-B mutant Δ747 (Yahiro *et al*., 2004) expressed in COS-7 cells was reduced to an extent similar to that of m1VacA by *O*-glycosidase treatment (data not shown), suggesting that *O*-linked glycosylation of RPTPβ-B affected directly or indirectly RPTPβ-B binding of m1VacA as well as m2VacA. Although we do not have any information about the chemical structure of *O*-linked glycan of RPTPα in HeLa and other cells susceptible to m2VacA, weak binding of m2VacA to HeLa cells may be resulting from the structural differences between HeLa cell RPTPα and RPTPα from other m2VacA-sensitive cells. In RK13 and AGS cells, which lack RPTPβ as judged by reverse transcription polymerase chain reaction (RT-PCR) (data not shown), much more RPTPα is present as compared with HeLa or AZ-521 cells (Fig. 6A). Furthermore, it appears that the RPTPα band from AGS cells has a slower mobility on SDS-polyacrylamide gels compared with RPTPα from other cells. The chemical structure of *O*-linked glycan of RPTPα in AGS may support its high affinity binding to both m1VacA and m2VacA as compared with glycosylated RPTPα from other cells susceptible to VacA. The difference in size was not resulting from a difference in RPTPα sequences of G401 and HeLa cells, which were found to be virtually identical to the published RPTPα sequence of the RPTPα isoform (Daum *et al*., 1994). The smaller size of RPTPα in HeLa cell might reflect a structural difference responsible for reduced binding of m2VacA, resulting in lower toxicity. In addition, it is well known that the internalization of VacA affects its vacuolating activity (Cover and Blanke, 2005). m1VacA and m2VacA are internalized with similar efficiency in G401 cells. In contrast, m2VacA was internalized much less readily, compared with m1VacA in HeLa cells, in agreement with its relatively low binding (Fig. 4).

Seto *et al*. (1998) reported that the epidermal growth factor (EGF) receptor functions as m1VacA receptor in HeLa cells. We did not confirm the fact that anti-EGFR antibody neutralized m1VacA-induced vacuolation and

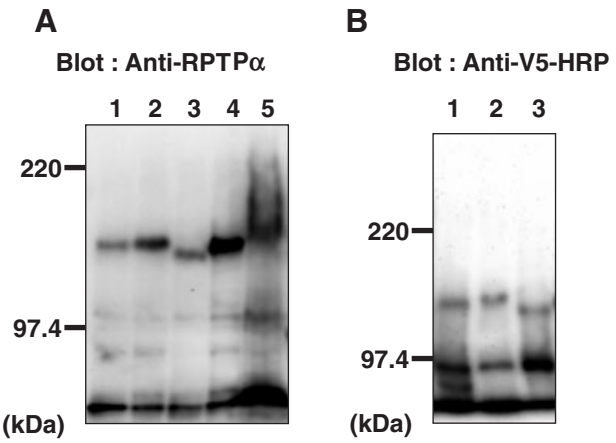

Fig. 6. Western Blot analysis of RPTPα in AZ-521, G401, HeLa, RK-13 and AGS cells.
A. Samples (1×10^7 cells) of AZ-521, G401, HeLa, RK-13 and AGS cells (lanes 1, 2, 3, 4 and 5 respectively) were lysed in SDS buffer and lysate proteins (10 μg) were separated by SDS-PAGE in 5% gels, followed by Western blotting with anti-RPTPα antibodies.
B. After transfection of COS-7, G401 and HeLa cells (lanes 1, 2 and 3 respectively) with 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 Data are representative of three separate experiments.

m1VacA binding to HeLa cells. These findings, together with the failure of anti-EGF receptor antibodies to interfere with binding of acid-activated m1VacA to HeLa cells, are consistent with the possibility that RPTPα is a major receptor for m1VacA and transmits the signal that induces vacuolation. However, RPTPα of HeLa cells is not a good receptor for m2 VacA when compared with RPTPα of other m2VacA-sensitive cells.

Experimental procedures

Bacterial strains and vacuolating cytotoxin production

Helicobacter pylori strains with the m2 mid-region were acquired from Fukui Medical School. The five strains, OK139 and OK160 associated with gastritis and OK187, OK204 and OK210, which were associated with duodenal ulcer plus ATCC49503, which has m1 genotype, were grown on blood agar plates (MH II plus 10% defribinated horse blood). By Western blotting using anti-VacA antibody raised against m1VacA**,** the presence of m2VacA in culture medium of *H. pylori* strains with m2 genotype was examined using 50% ammonium sulphate precipitate of culture supernatant from each strain.

Nucleotide sequence of the entire vacA gene

DNA from *H. pylori* strains, OK160 and OK210, was extracted from the pellet of the bacterial suspension. Primers for PCR amplification and direct sequencing of the entire coding region of *vacA* are shown in Table 1. After PCR products were purified, direct DNA sequencing was performed. The previously published *vacA* gene sequences of strain 95–54 (GenBank Accession number HPU95971) and strain Tx30a (HPU29401) were also included in the analysis.

Purification of m1VacA and m2VacA

m2VacA from the supernatant of the OK210 strain was precipitated with a 50% saturated solution of ammonium sulphate. The protein in the precipitate was further purified by sequential column chromatography. The dialysed sample was applied to a hydroxyapatite column (Econo-Pac cartridge CHT-II column (Bio-Rad) equilibrated with 10 mM sodium phosphate, pH 7.2. After washing the column with same buffer, m2VacA was eluted with two linear gradients of 10 mM to 300 mM and 300 mM to 600 mM sodium phosphate, pH 7.2. Fractions that reacted with anti-m1VacA IgG were pooled and subjected to FPLC using a Superose 6 HR 10/30 column (Amersham Pharmacia Biotech), equilibrated with 10 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl. The eluate was monitored for absorbance at 280 nm and by Western blotting using the anti-m1VacA IgG. m1VacA was purified from strain 60190 using an m1VacA affinity matrix prepared by coupling CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) with anti-VacA-specific IgG antibody, and equilibrated with RX buffer (10 mM KCl, 0.3 mM NaCl, 0.35 mM $MgCl_2$ and 0.125 mM EGTA in 1 mM HEPES, pH 7.3).

Table 1. Primers used for sequencing of m2 VacA DNA.

Primer	Nucleotide sequence[a]	Corresponding DNA sequence (size of PCR product)
VAS-1F	5′-AGC CGA TAG CAT CAG AGA AGA AC-3′	95–117[b]
VAS-11R	5′-TGT GGT GTA TGC GTT GTA GGG GTT-3′	4375–4352[b]
vacA s1a-F*	5′-CTC TCG CTT TAG TAG GAG C-3′	452–470[b]
vacA s1c-F*	5′-CTC TCG CTT TAG TGG GGY T-3′	
vacA S5*	5′-GCT AAC CGC ACC ACG AGA GT-3′	959–968[b]
vacA AS5*	5′-TTG TCT GTA ACG CCG CTA AAA-3′	2009– 1999[b]
VA4-F*	5′-GGA GCC CCA GGA AAC ATT G-3′	976–994[c]
vacA AS4*	5′-ATA ACT AGC GCC TTG CAC TC-3′	1326–1307[c]
vacA S3*	5′-TAT TGA AAG CGT GTT TGA AT-3′	3069–3088[b]
vacA-3′F*	5′-CAT TGT GGG CGG TTT TGG AAG-3′	4156–4177[b]

a. Y is C or T.
b. Nucleotide positions in the VacA gene of *H. pylori* 11638 (GenBank Accession no. HPU07145) (Phadnis *et al*., 1994; Ito *et al*., 1998).
c. Nucleotide positions in the VacA gene of *H. pylori* 87–203 (GenBank Accession no. HPU05677) (Cover *et al*., 1994; Atherton *et al*., 1995).
*Primer used only for DNA sequencing.

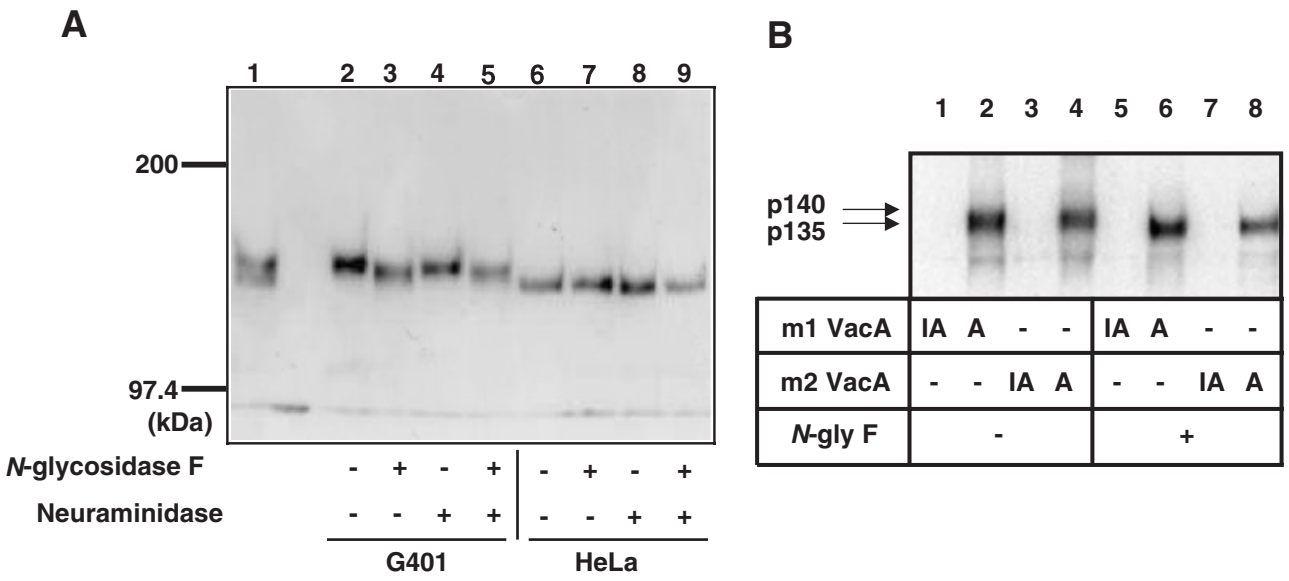

Fig. 7. Comparison of molecular sizes and VacA binding activity of RPTPα between G401 and HeLa cells after treatment with *N*-glycosidase F.
A. Molecular size of RPTPα from G401 and HeLa cells after incubation with *N*-glycosidase F and neuraminidase. Solubilized proteins (200 μg) from G401 or HeLa cells were incubated at 37°C for 3 h without or with *N*-glycosidase F (0.5 U), and neuraminidase (0.1 U) as indicated, before samples (20 μg) were separated by SDS-PAGE in 5% gels, followed by Western blotting using anti-RPTPα antibody. Lane 1 contains a mixture (15 μg of protein) of G401 cell lysate incubated with or without *N*-glycosidase F and HeLa cell lysate to confirm each band of native or hydrolysed RPTPα in the same lane.
B. Immunoprecipitation of RPTPα of G401 cells with m1VacA and m2VacA after treatment with *N*-glycosidase F. Proteins solubilized from biotinylated cells were incubated without (lanes 1, 2, 3 and 4) or with *N*-glycosidase F (*N*-gly F, lanes 5, 6, 7 and 8), at 37°C for 3 h. Samples were then incubated with heat-inactivated form (IA) or native form (A) of m1VacA (lanes 1, 2, 5 and 6) and m2VacA (lanes 3, 4, 7 and 8) before immunoprecipitation with anti-VacA antibodies and SDS-PAGE in 5% gels followed by transfer to PVDF membranes; untreated RPTPα (p140) and *N*-glycosidase F-treated RPTPα (p135) were visualized by incubation with HRP-conjugated streptavidin followed by ECL detection. Arrows show the location of p140 and p135 respectively. Data are representative of three separate experiments.

After washing the column with RX buffer, m1VacA was eluted with 50 mM glycine-HCl buffer, pH 1, which was then neutralized with 1 M Tris. After gel filtration on a Superose 6 HR 10/30 column equilibrated with TBS buffer (10 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl), purified m1VacA was concentrated and stored (200 μg ml^{-1}). m1VacA purified using the m1VacA affinity column is in the acid-activated form.

Assay for vacuolating activity

Quantification of cell vacuolation by the purified m2VacA was performed by the NRUassay (Cover *et al*., 1991) AZ-521 (Culture Collection of Health Science Resources Bank, Japan Health Science Foundation), HeLa and RK13 cells were grown in EMEM containing 10% fetal calf serum (FCS); G-401 cells were grown in Dulbecco's modified Eagle's medium containing 10% FCS. Cells were seeded in 96-well culture plates (2×10^4 cells in 100 μl/well) and grown for 24 h in a 5% CO_2 atmosphere at 37°C. Purified VacA was added to a final concentration of 120 nM and cells were incubated for an additional 8 h at 37°C. To evaluate the effect of acid- or alkali-treatment on vacuolating activity, m2VacA solutions were adjusted to pH 1.5 or 11.5 by the addition of 1N HCl or 1N NaOH, respectively, incubated at 30°C for 10 min and then neutralized to pH 7.7 with 1N NaOH or 1N HCl respectively. The resulting samples were added to cells, followed by incubation for indicated times. To evaluate the effect of anti-EGFR antibody (MONOSAN, the Netherlands), HeLa cells were incubated with anti-EGFR antibody (2.5–10 μg ml^{-1}) for 30 min at 37°C, followed by incubation with 120 nM activated m1VacA for an additional 8 h at 37°C before the NRU.

Immunoprecipitation

Immunoprecipitation of m2VacA receptors was performed as reported by Yahiro *et al*. (1999). Cultured cells were harvested with TNE buffer and washed twice with PBS. Protein on the cell surface was biotinylated according to manufacturer's specification (Amersham Pharmacia Biotech; ECL protein biotinylation module, RPN 2202) and cells were lysed with 1 ml of Sol Buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10% glycerol, 5 μg leupeptin and 1% Triton X-100) for 15 min on ice. After centrifugation (20 min, 15000 *g*), the supernatant (300 μl) was incubated at 4°C for 1 h with 1 μg of inactivated (100°C for 10 min), acid- or alkali-activated VacA toxin. The mixture was then incubated at 4°C overnight with rabbit antibodies raised against purified VacA or a glutathione-S-transferase (GST)-fusion protein containing the entire cytoplasmic domain of murine RPTPα (Jiang *et al*., 1999). Antibody-bound proteins were collected after addition of protein-A-Sepharose CL-4B (Amersham Pharmacia Biotech) in Sol buffer, and incubation at 4°C for 1 h. After beads were washed with Sol buffer, proteins were solubilized by heating in SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 1.6% SDS, 8% glycerol, 2% dithiothreitol and 0.08% bromphenol blue), separated by SDS-PAGE in 5% gels, and transferred to polyvinylidine difluoride

(PVDF) membranes. Blots were incubated with streptavidin-horseradish peroxidase (streptavidin-HRP), anti-VacA antibody, or anti-RPTPβ monoclonal antibody (Transduction lab) and then with peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG antibody. Proteins were detected using ECL.

RNA preparation and RT-PCR

Total RNA (0.5 μg) was extracted from AZ-521, G401, HeLa, AGS and RK13 cells by using ISOGEN (Nippon gene, Tokyo) and reverse transcribed into single-strand cDNA (1st strand cDNA synthesis Kit, Roche) using Oligo dT primers for RPTPβ-B, which is a short receptor form of RPTPβ, or random primer for RPTPα. The resulting cDNA (0.25 μg) was used as a template for PCR to amplify specific cDNA regions of human RPTPα or human RPTPβ-B (short form of human RPTPβ) by using primers RPTPα-fwd (5′-GGGTCAGCATGGATTCCTGGTTCATTCTT-3′), and RPTPα-rev (5′-CCAAGCTTATTGCTTGTATTTCTTAAA CCT), RPTPβ-Bfwd (5′-GAAGATTCAACTTCATCAGGTTCA GAA-3′), RPTPβ-rev (5′-ACCCTGCTATGATCATAGGCAACGAT-3′) (Yahiro *et al*., 2004). After initial denaturation for 1 min at 95°C, 30 cycles of denaturation (30 s, 95°C), annealing (30 s, 60°C), and elongation (1 min, 72°C) were followed by a final elongation for 7 min at 72°C. For control amplifications of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, similar cycling conditions were used.

Flow cytometric analysis of toxin binding

HeLa or G401 cells (1×10^7), harvested with TNE buffer, were washed twice with PBS and suspended in 1 ml of PBS containing 2% bovine serum albumin (BSA). The suspension (100 μl) was mixed with 120 nM native, acid-activated, or heat-inactivated m2VacA at 4°C for 30 min. As a control, cells were incubated with activated m1VacA, heat-inactivated m1VacA, or without toxin. After cells were washed with PBS containing 2% BSA, anti-m1VacA antibody was added, followed by incubation at 4°C for 30 min. Cells were then washed, suspended in PBS containing 2% BSA and incubated at 4°C for 30 min with Alexa Fluor ^{TM488}-labelled goat anti-rabbit IgG (H + L) 1:400 (Molecular Probes). After washing with PBS containing 2% BSA, samples were analysed in a FACScan flow cytometer (Becton Dickinson Immunocytometry System, CA, USA) with excitation at 488 nm and emission at 530 nm.

Expression of RPTPa in G401 and HeLa cells

cDNA for human RPTPα in a pcDNA3.1 vector (purchased from Invitrogen) has a V5 epitope at the C terminus. COS-7, G401 and HeLa cells were plated 24 h before transfection, which was performed using GenePorter transfection reagent (Gene Therapy System), following the manufacturer's protocol. After transfection, cells were incubated at 37°C for 48 h, harvested in TNE buffer, washed with ice-cold PBS buffer, lysed in Sol buffer, and centrifuged at 15 000 *g* for 20 min. The supernatant was incubated at 4°C for 1 h with 0.5 μg of native or heat-inactivated VacA. The mixtures were then incubated at 4°C overnight with 1 μl of an antibody raised against purified VacA. After addition of 25 μl of protein A-Sepharose CL-4B (Amersham Biosciences), 0.1 g ml^{-1} in Sol buffer, and incubation at 4°C for 1 h, beads were washed three times with Sol buffer. Bound proteins were solubilized by heating in 5 μl of SDS-PAGE sample buffer, separated by SDS-PAGE in 5% gels, and transferred to PVDF membranes. Blots were incubated with HRP-conjugated anti-V5 monoclonal antibody (Invitrogen), which recognizes the 14-amino acid (GKPIP-NPLLGLDST) sequence of V5, and proteins were detected using the ECL system.

RPTPα sequences of G401 or HeLa cells

The RNA was extracted from G401 or HeLa cells using ISOGEN (Nippongene, Tokyo) and reverse transcribed using GeneAmp RNA PCR Core Kit (Roche). The resulting cDNA was used as a template for PCR to amplify specific RPTPα cDNA regions. Primers RPTPα-696S(5′-GGGCTAGCATGGATTCCTGGTTC ATTCTT-3′), RPTPα-1401AS(5′-CCAAGCTTATTGCTTGTATTT CTTAAACCT-3′), RPTPα-1141S(5′-AGACACCAATTATTGCG GTGA-3′), RPTPα-2161AS(5′-GGTATATGAAGACATACTGCA-3′), RPTPα-2101S(5′-ACGTGTATGGCTTTGTGAGCC-3′) and RPTPα-3101AS(5′-CGGACCCTTGTTGCCGCTTAC-3′. These fragments were subcloned into the pBluescript vector. DNA sequences of these fragments were analysed using the DNA Sequencing Kit BigDye and ABI PRISM 310 Genetic Analyzer.

Other methods and chemicals

Protein was measured by the method of Bradford using BSA as standard (Bradford, 1976). Molecular mass standards for SDS-PAGE were myosin (220 kDa), rabbit muscle phosphorylase *b* (97.4 kDa), BSA (66 kDa) and ovalbumin (45 kDa). Other reagents were of analytical grade.

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