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A 12-Amino-Acid Segment, Present in Type s2 but Not Type s1 *Helicobacter pylori* VacA Proteins, Abolishes Cytotoxin Activity and Alters Membrane Channel Formation

MARK S. MCCLAIN,[1] PING CAO,[1] HIDEKI IWAMOTO,[2] ARLENE D. VINION-DUBIEL,[3] GABOR SZABO,[2] ZHIFENG SHAO,[2] AND TIMOTHY L. COVER[1,3,4]*

Department of Medicine[1] and Department of Microbiology and Immunology,[3] Vanderbilt University School of Medicine, and Department of Veterans Affairs Medical Center,[4] Nashville, Tennessee, and Department of Molecular Physiology and Biological Physics and Biophysics Program, University of Virginia School of Medicine, Charlottesville, Virginia[2]

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***Helicobacter pylori*, a gram-negative bacterium associated with gastritis, peptic ulceration, and gastric adenocarcinoma in humans, secretes a protein toxin, VacA, that causes vacuolar degeneration of epithelial cells. Several different families of *H. pylori vacA* alleles can be distinguished based on sequence diversity in the "middle" region (i.e., m1 and m2) and in the 5′ end of the gene (i.e., s1 and s2). Type s2 VacA toxins contain a 12-amino-acid amino-terminal hydrophilic segment, which is absent from type s1 toxins. To examine the functional properties of VacA toxins containing this 12-amino-acid segment, we analyzed a wild-type s1/m1 VacA and a chimeric s2/m1 VacA protein. Purified s1/m1 VacA from *H. pylori* strain 60190 induced vacuolation in HeLa and Vero cells, whereas the chimeric s2/m1 toxin (in which the s1 sequence of VacA from strain 60190 was replaced with the s2 sequence from strain Tx30a) lacked detectable cytotoxic activity. Type s1/m1 VacA from strain 60190 formed membrane channels in a planar lipid bilayer assay at a significantly higher rate than did s2/m1 VacA. However, membrane channels formed by type s1 VacA and type s2 VacA proteins exhibited similar anion selectivities (permeability ratio, $P_{Cl}/P_{Na} = 5$). When an equimolar mixture of the chimeric s2/m1 toxin and the wild-type s1/m1 toxin was added to HeLa cells, the chimeric toxin completely inhibited the activity of the s1/m1 toxin. Thus, the s2/m1 toxin exhibited a dominant-negative phenotype similar to that of a previously described mutant toxin, VacA-(Δ6–27). Immunoprecipitation experiments indicated that both s2/m1 VacA and VacA-(Δ6–27) could physically interact with a c-myc epitope-tagged s1/m1 VacA, which suggests that the dominant-negative phenotype results from the formation of heterooligomeric VacA complexes with defective functional activity. Despite detectable differences in the channel-forming activities and cytotoxic properties of type s1 and type s2 VacA proteins, the conservation of type s2 sequences in many *H. pylori* isolates suggests that type s2 VacA proteins retain an important biological activity.**

Helicobacter pylori is a gram-negative bacterium that colonizes the gastric mucosa of humans. Colonization with these organisms consistently induces gastric mucosal inflammation and is associated with an increased risk for peptic ulcer disease, gastric adenocarcinoma, and gastric lymphoma (6, 16).

The only cytotoxin known to be secreted into the extracellular space by *H. pylori* is the vacuolating cytotoxin, VacA (5, 37). The hallmark of VacA activity is the formation of prominent intracellular vacuoles when the toxin is added to cultured cells (30). These vacuoles represent hybrid compartments derived from late endosomes and lysosomes (35). The mechanism of VacA-induced vacuole formation is not yet completely understood but is thought to involve alterations in membrane trafficking along the endosomal-lysosomal pathway (37) and seems to be dependent on the formation of anion-selective channels in cellular membranes (11, 24, 53, 56, 57). One current model suggests that vacuolation is somehow related to an influx of anions through VacA channels formed in the membranes of endosomes (11, 24, 53, 56, 57). In addition to causing formation of intracellular vacuoles, VacA interferes with the process of antigen presentation (36), increases the permeability of polarized epithelial monolayers (42), induces apoptosis (18, 43), and interacts with a cellular protein associated with intermediate filaments (13). The results of these studies suggest that VacA is a multifunctional toxin.

The *vacA* gene encodes a 140-kDa precursor protein which is cleaved at both its N and C termini to yield the mature 88-kDa secreted VacA cytotoxin monomer (7, 10, 38, 48, 55). These 88-kDa monomers assemble into complex flower-shaped oligomeric structures (8, 31). Upon exposure to acidic or alkaline pH, VacA oligomers dissociate into the component monomers, which are capable of reassembling into oligomeric structures under neutral-pH conditions (8, 34, 62). Exposure of the purified oligomeric toxin to acidic or alkaline pH (activation) results in enhanced internalization of the toxin by cells and markedly increases its cytotoxic activity (14, 33).

There is a high level of sequence diversity among *vacA* genes from different *H. pylori* strains, and several families of *vacA* alleles are recognized (1). Two families (s1 and s2) can be differentiated based on analysis of sequences at the 5′ end of the *vacA* gene, including the portion that encodes the VacA amino-terminal signal sequence, and two additional families (m1 and m2) can be differentiated based on analysis of *vacA*

* Corresponding author. Mailing address: Division of Infectious Diseases, A3310 Medical Center North, Vanderbilt University School of Medicine, Nashville, TN 37232. Phone: (615) 322-2035. Fax: (615) 343-6160. E-mail: COVERTL@ctrvax.vanderbilt.edu.

TABLE 1. Bacterial strains and plasmids used in this study

H. pylori strain or plasmid	Genotype or description	Reference
60190 (ATCC 49503)	*vacA* s1/m1	10
Tx30a (ATCC 51932)	*vacA* s2/m2	1
VM022	60190 with *sacB-kan* replacing *Bsm*FI-to-*Eco*NI region of *vacA*	61
VM083	*vacA* s2/m1; VM022 with *vacA* gene restored, including the s2 region from pA177	This study
VM084	*vacA* s1/m1; VM083 transformed with pAV202	This study
VT330	*vacA* s1/m1-c-myc; VM022 with *vacA* gene restored, including the c-myc epitope from plasmid pVT330	This study
AV452	Expresses VacA-(Δ6-27)	61
Plasmids		
pA148	6.3-kb *Eco*RV-*Xho*I fragment containing the entire *vacA* gene from *H. pylori* strain 60190 cloned into pBluescript	3
pA167	pA148 digested with *Kpn*I and *Bgl*II; ends blunted and religated	This study
pA176	*Stu*I site introduced into pA167 at *vacA* codons 12 and 13	This study
pA177	*Stu*I-to-*Bcl*I fragment of pA176 replaced with *Stu*I-to-*Bcl*I-digested *vacA* PCR product from *H. pylori* Tx30a	This study
pAV202	Derivative of *H. pylori* strain 60190 VXC-1 (17) containing nucleotides 49 through 1679 of GenBank accession no. U05676 (including 883 nucleotides from the 5′ end of *vacA*) and containing a chloramphenicol resistance determinant in the *cysS-vacA* intergenic region	This study
pA178	Site-specific mutagenesis of pA167, resulting in introduction of a *Stu*I restriction site at *vacA* codons 349 to 351	This study
pVT330	Oligonucleotides encoding the c-myc epitope inserted into the *Stu*I site of pA178	This study

"midregions" (1). Various s1, m1, and m2 subfamilies of *vacA* alleles have also been described (1, 22, 51). Analysis of *H. pylori* isolates from multiple unrelated persons indicates that recombination among *vacA* alleles has occurred commonly (52), but the main families of *vacA* sequences (s1, s2, m1, and m2) have nevertheless remained relatively intact. This suggests that various in vivo selective forces favor preservation of these structures.

The classification of *vacA* alleles according to families, particularly according to s1 or s2 types, seems to correlate with the risk for clinical disease. Numerous studies have concluded that peptic ulceration occurs more commonly among patients infected with *H. pylori* strains containing a type s1 *vacA* allele than among patients infected with strains containing a type s2 *vacA* allele (1, 15, 19, 22, 27, 45, 51, 59). This association is less apparent in many Asian countries than in Europe and the Americas (41). To account for the association of certain *vacA* genotypes with peptic ulcer disease in Western countries, at least three possible explanations have been suggested. First, strains that contain type s1 *vacA* alleles more frequently contain the *cag* pathogenicity island and more frequently express the BabA2 adhesin (a Lewis-b binding factor) than do strains that contain type s2 *vacA* alleles, which suggests that multiple bacterial factors could contribute to ulcerogenesis (1, 19, 39, 45). In particular, products of the *cag* pathogenicity island contribute to induction of a gastric mucosal inflammatory response, which seems to play an important role in the pathogenesis of peptic ulcer disease (39, 44, 45). Second, strains that contain type s1 *vacA* alleles transcribe and express higher levels of VacA than do strains with type s2 *vacA* alleles (17), and type s1 VacA signal sequences may be more efficient than type s2 signal sequences in transporting the VacA protoxin across the cytoplasmic membrane. Finally, it is possible that mature VacA proteins containing a type s1 amino terminus cause gastric epithelial damage to a greater extent than do VacA proteins containing a type s2 amino terminus (1, 17, 28). Gastric epithelial damage induced by type s1 VacA may contribute to the pathogenesis of peptic ulceration (20, 55).

Several studies have suggested that type s2 VacA proteins are relatively noncytotoxic in vitro compared to type s1 VacA proteins (1, 17, 28). In one recent study, Letley and Atherton constructed a recombinant *H. pylori* strain that secreted a chimeric s2/m1 VacA protein and reported that broth culture supernatant from this strain lacked cytotoxic activity (28). The functional properties of type s2 VacA toxins have not yet been examined in any detail, in part because these proteins are typically secreted at low levels by wild-type *H. pylori* strains and are thus difficult to purify in reasonable quantities. In this study, we tested the hypothesis that type s1 and type s2 VacA proteins differ in the capacity to form anion-selective membrane channels. We report that a 12-amino-acid hydrophilic amino-terminal segment, present in type s2 but absent from type s1 VacA proteins, diminishes the capacity of VacA to form membrane channels and induce cytotoxic effects. In addition, we report that an s2/m1 chimeric toxin can inhibit the vacuolating activity of wild-type s1/m1 VacA and thus exhibits a dominant-negative phenotype.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *H. pylori* strains 60190 (ATCC 49503) and Tx30a (ATCC 51932) contain prototypes for two highly divergent families of *vacA* alleles (designated type s1/m1 and type s2/m2, respectively) (1, 10). *H. pylori* cells were grown on Trypticase soy agar plates containing 5% sheep blood at 37°C in ambient air containing 5% CO_2. Liquid cultures were grown in sulfite-free brucella broth containing either 5% fetal bovine serum or 0.5% activated charcoal.

VacA purification. VacA was purified from *H. pylori* broth culture supernatants as described previously (8), except that the buffer was phosphate-buffered saline (PBS) (pH 7.5) containing 1 mM EDTA and 0.02% sodium azide. Briefly, broth culture supernatant proteins were concentrated by precipitation with a 50% saturated solution of ammonium sulfate, and the oligomeric form of VacA was isolated by fractionation using a Superose 6 HR 16/50 gel filtration column.

Cell culture. HeLa and Vero cells were grown in minimal essential medium (modified Eagle's medium containing Earle's salts; MEM) containing 10% fetal bovine serum. Serial dilutions of concentrated *H. pylori* broth culture supernatants or purified VacA were incubated with cultured cells in a microtiter format, as described previously (9). Purified VacA preparations were routinely acid activated by adjusting them to pH 3 by the addition of 250 mM hydrochloric acid before they were added to cell culture wells (8, 14). After incubation for 24 h, the cells were examined by inverted light microscopy. Samples that induced vacuolation in >50% of the cells were scored as positive for the vacuolating cytotoxin phenotype (1). In some experiments, vacuolation was also quantified by neutral red uptake assay (9).

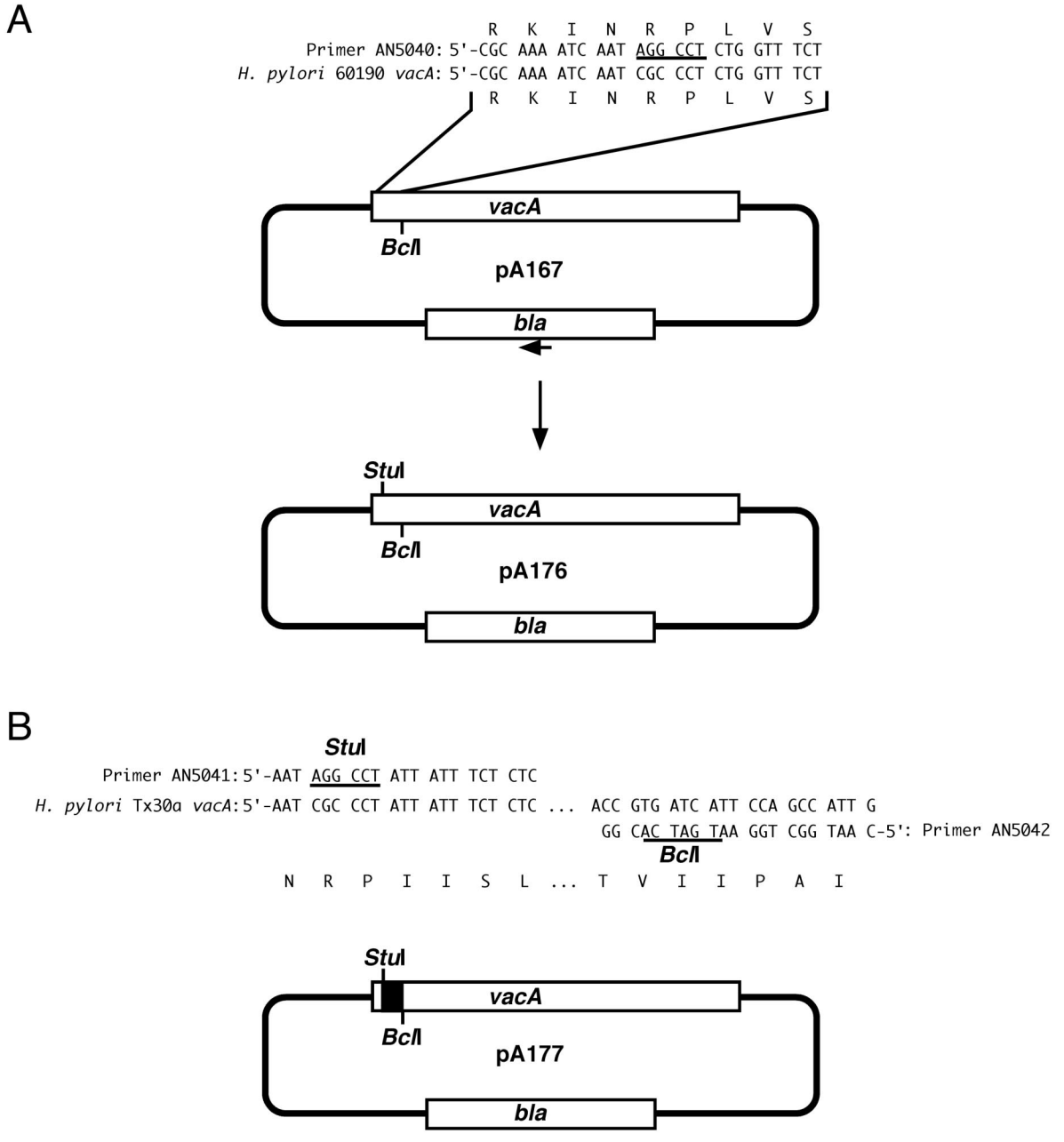

FIG. 1. Construction of plasmids used to generate *H. pylori* strain VM083. (A) A *Stu*I restriction site (underlined) was introduced into plasmid pA167 using oligonucleotide primer AN5040 and the selection oligonucleotide (small arrow) from the Gene Editor kit (Promega) to generate plasmid pA176, as described in Materials and Methods. (B) A 130-nucleotide *vacA* fragment containing an s2 *vacA* sequence was PCR amplified from *H. pylori* strain Tx30a using primers AN5041 and AN5042. The 111-nucleotide *Stu*I-to-*Bcl*I restriction fragment from this PCR product (solid box) was introduced into pA176 to generate pA177. Natural transformation of *H. pylori* VM022 with pA177 yielded *H. pylori* strain VM083. *H. pylori* VM083 expresses a chimeric s2/m1 VacA toxin.

Introduction of a type s2 *vacA* sequence into *H. pylori* 60190. We first constructed a plasmid (pA167) that contains an ≈3-kb fragment including the 5′ end of *vacA* from *H. pylori* strain 60190 (Table 1). A *Stu*I site was introduced into pA167 at a site encoding amino acids 12 and 13 of the VacA signal sequence, thereby generating plasmid pA176 (Fig. 1). This was accomplished using the Gene Editor site-directed mutagenesis kit (Promega) with oligonucleotide AN5040 (5′-CGCAAAATCAATAGGCCTCTGGTTTCT) and a selection oligonucleotide as described in the kit. A 130-nucleotide *vacA* fragment containing an s2 sequence was then PCR amplified from *H. pylori* Tx30a using primers AN5041 (5′-AATAGGCCTATTATTTCTCTC) and AN5042 (5′-CAATGGCT GGAATGATCACGG). Both the PCR product and pA176 were digested with *Stu*I and *Bcl*I, and thereafter, the s2-containing segment from *H. pylori* Tx30a was cloned into the digested pA176 to yield pA177 (Fig. 1). *H. pylori* VM022, which contains a *sacB-kan* cassette within *vacA* (4, 61), was then transformed with pA177, and a sucrose-resistant, kanamycin-sensitive colony was selected. PCR and DNA sequence analysis confirmed that a double-crossover event had occurred in the chromosome of this strain (VM083) and that the strain now contained a chimeric s2/m1 *vacA* allele. To restore the original type s1 *vacA* sequence, strain VM083 was transformed with pAV202 (containing a fragment of *vacA* from *H. pylori* 60190, described in Table 1), and chloramphenicol-resistant colonies were selected. PCR and DNA sequence analysis of one such colony (VM084) confirmed that a type s1 *vacA* sequence, identical to that in wild-type strain 60190, was present.

Amino-terminal sequence analysis. VacA purified from culture supernatant of *H. pylori* strain VM083 was electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride paper (Bio-Rad) by electroblotting it for 30 min at 50 V in 10 mM 3-cyclohexylamino-1-propanesulfonic acid buffer (pH 11). After Coomassie blue staining, the VacA band was excised, and amino-terminal sequence analysis was performed with a PE Biosystems Procise 492 protein sequencing apparatus in the Peptide Sequencing and Amino Acid Analysis Shared Resource of the Vanderbilt University School of Medicine.

Introduction of DNA encoding a c-myc epitope tag into the *vacA* allele of *H. pylori* 60190. To facilitate the expression of an epitope-tagged form of VacA, a unique *Stu*I restriction site was first introduced using the Gene Editor site-directed mutagenesis kit (Promega) into plasmid pA167 to generate plasmid pA178 (Table 1). (Introduction of the *Stu*I restriction site was accompanied by missense mutations at codons 312, 314, 315, and 317, replacing each of the wild-type codons with alanine-encoding codons.) Complementary primers (AN5341 [5′-CCGAACAGAAACTGATATCTGAAGAAGATCTAG] and AN5342 [5′-CTAGATCTTCTTCAGATATCAGTTTCTGTTCGG]) encoding the c-myc epitope (EQKLISEEDL) were annealed and ligated into the unique *Stu*I site of plasmid pA178. Sequence analysis of the resulting plasmid, pVT330 (Table 1), revealed a single copy of the c-myc epitope-encoding sequence in the proper orientation. Plasmid pVT330 was then transformed into *H. pylori* strain VM022. Transformants in which the *sacB-kan* cassette was replaced by a *vacA*–c-*myc* sequence were selected by growth on plates containing 7.5% sucrose. Sequence analysis of PCR-amplified DNA from one of the transformants (*H. pylori* strain VT330) (Table 1) verified that the *vacA* allele now encoded a c-myc epitope. The predicted amino acid sequence of VacA residues 310 to 320 produced by *H. pylori* strain VT330 is as follows (the c-myc epitope is underlined): GAANAAQAEQKLISEEDLASSQ.

Biotinylation of VacA. Purified VacA was concentrated, and the buffer was exchanged with 25 mM HEPES (pH 7.2) using Centriprep 30 centrifugal concentrators (Amicon). The protein concentration was determined using the Micro BCA protein assay (Pierce). Aliquots of the concentrated toxin were placed in microcentrifuge tubes containing a 1/10 volume of sodium bicarbonate (7.5% [wt/vol]). *N*-Hydroxysuccinimidobiotin (NHS-biotin; Pierce) dissolved in dimethyl sulfoxide was added at molar ratios of NHS-biotin to VacA ranging from 1:1 to 5:1, and the reaction mixtures were incubated at 25°C for 1 h. The reactions were stopped by addition of a 1/10 volume of hydroxylamine (10 mg per ml). The biotinylated toxin was then separated from unincorporated biotin by gel filtration chromatography using a Micro Bio-Spin chromatography column (Bio-Rad) containing Bio-Gel P-6 equilibrated in 25 mM HEPES containing 150 mM sodium chloride and bovine serum albumin (100 μg per ml). Under these conditions, biotinylation of VacA from *H. pylori* strain 60190 resulted in minimal loss of vacuolating activity. Biotinylated VacA was detected in immunoblotting studies by use of streptavidin-conjugated horseradish peroxidase (Life Technologies) and enhanced chemiluminescence (Amersham Pharmacia Biotech).

Immunoprecipitations of c-myc-tagged VacA. Equimolar mixtures of biotinylated and c-myc-tagged VacA were diluted in 1 ml of PBS (pH 7) containing 0.05% Tween 20 and 2% ammonium sulfate (PBS-T-AS) to yield a final concentration of 2 μg of each toxin species per ml. When necessary, the toxins were adjusted to pH 3 (acid activated) by dropwise addition of 250 mM HCl and then neutralized upon dilution in PBS-T-AS. Anti-c-myc monoclonal antibody (9E10; Roche) (5 μg) was added to the VacA preparation, and the mixture was incubated at 4°C for 1 h. Protein G-Sepharose beads (Zymed) (25 μl), washed twice with PBS-T-AS, were added to the toxin-antibody mixture and incubated for an additional hour at 4°C. The beads were then washed three times in PBS-T-AS. The immunoprecipitated proteins were separated from the beads by boiling the beads in SDS-PAGE sample buffer and were analyzed by immunoblot analysis.

Electrophysiologic analysis of VacA channel-forming activity. Planar lipid bilayers, composed of egg phosphatidylcholine-dioleoylphosphatidylserine-cholesterol (55:15:30 mol%) dissolved in *n*-decane, were prepared as described previously (11, 24, 61). Purified acid-activated VacA toxins were added to the lipid bilayers in a buffer consisting of 5 mM citric acid (pH 4.0) and 2 mM EDTA, with the salt composition as described in the figure legends and tables. For experiments using mixtures of different VacA proteins, the two VacA species (each 30 nM) were mixed together at neutral pH, and the mixture was then acidified to pH 3 and maintained at this pH for 1 h before being added to planar lipid bilayers. Membrane currents were measured as described previously (61). The potential is indicated relative to the *cis* side, defined as the chamber to which the protein was added. Permeability ratios were determined from the Goldman-Hodgkin-Katz equation (21), after the membrane voltage for zero current (reversal potential) in asymmetric salt concentrations was measured. Statistical significance was analyzed using Student's *t* test.

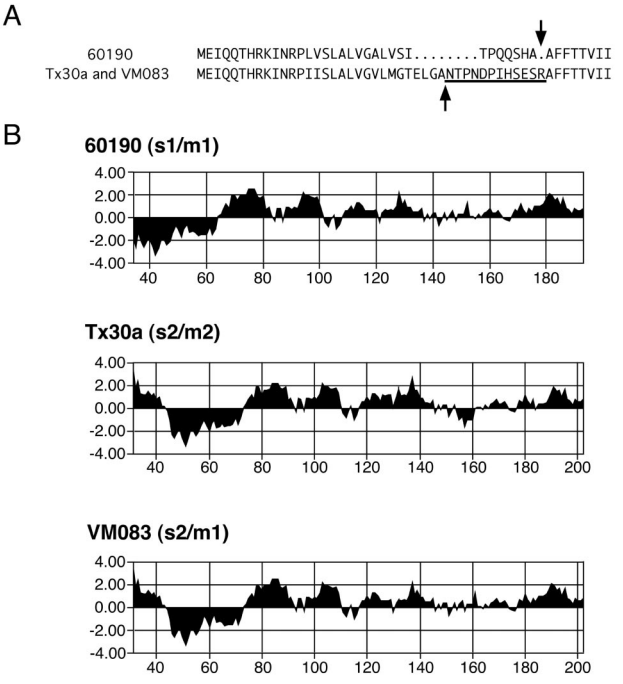

FIG. 2. Sequence diversity in the amino termini of s1- and s2-type toxins. (A) Amino-terminal amino acid sequences of type s1 (from *H. pylori* strain 60190) and s2 (from *H. pylori* strains Tx30a and VM083) VacA toxins. The arrows indicate the sites at which the amino-terminal signal sequences are cleaved. The 12-amino-acid amino-terminal extension found in s2-type toxins is underlined. (B) Predicted hydrophilicity of VacA toxins produced by *H. pylori* strains 60190, Tx30a, and VM083. The analysis is limited to the amino-terminal portion of each mature toxin. The amino acid numbering is based on the initiating methionine of the protoxins as amino acid 1. The 12-amino-acid amino-terminal extension found in s2 toxins is predicted to increase the hydrophilicity of the VacA amino terminus.

RESULTS

Construction and cell culture analysis of a chimeric s2/m1 VacA. Culture supernatants from wild-type *H. pylori* strains expressing type s1 VacA toxins induce vacuolation of HeLa and Vero cells, whereas supernatants from wild-type strains containing s2 VacA proteins lack vacuolating activity for these cell types (1, 17). To test experimentally whether the presence of a type s2 VacA sequence diminshes toxin activity, we introduced a 111-nucleotide type s2 sequence (derived from *H. pylori* strain Tx30a) into the *vacA* allele of *H. pylori* 60190, as described in Materials and Methods, thereby replacing the type s1 sequence with a type s2 sequence. An *H. pylori* strain (VM083) containing this chimeric s2/m1 *vacA* sequence expressed and secreted VacA at a level similar to that of wild-type *H. pylori* 60190, and the chimeric toxin could be purified as large oligomeric structures with a molecular mass greater than 900 kDa. Amino-terminal sequence analysis of the mature secreted s2/m1 chimeric protein from strain VM083 revealed that the protein underwent cleavage of a 30-amino-acid amino-terminal signal sequence (Fig. 2A). The site of signal sequence cleavage was different from that used in the wild-type strain 60190 but identical to that used in wild-type strain Tx30a (Fig. 2A) (1, 10). Notably, the mature secreted forms of both the chimeric s2/m1 VacA protein and the s2/m2 VacA protein

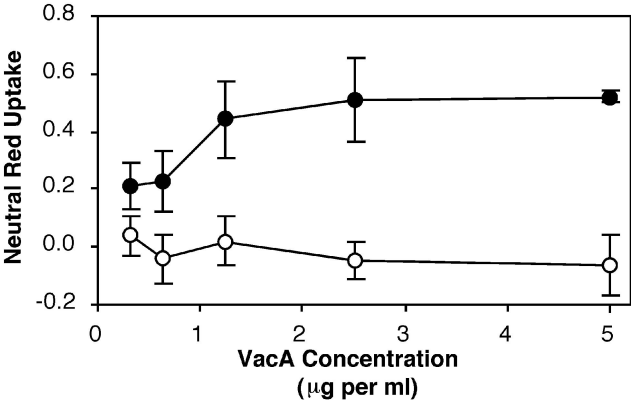

FIG. 3. Vacuolating activities of VacA toxins from strains 60190 (s1/m1) and VM083 (s2/m1). Purified, acid-activated VacA preparations from *H. pylori* strains 60190 (●) and VM083 (○) were incubated with HeLa cells in MEM containing 10 mM ammonium chloride for 16 h at 37°C. Vacuolating activity was quantified using a neutral red uptake assay (9). The results represent the mean (± standard deviation) net absorbance at 540 nm from triplicate samples. VacA from strain 60190 induced cell vacuolation, whereas the chimeric s2/m1 VacA from strain VM083 did not.

produced by wild-type strain Tx30a contain a 12-amino-acid extension (NTPNDPIHSESR) at the amino terminus compared with the amino-terminal sequence of wild-type s1/m1 VacA from *H. pylori* 60190 (Fig. 2A). This results in a marked change in the predicted hydrophobicity of this region, such that mature secreted type s2 VacA proteins contain a hydrophilic amino terminus whereas type s1 VacA proteins contain a hydrophobic amino terminus (Fig. 2B).

We next compared the cytotoxic activities of wild-type 60190 VacA and the chimeric s2/m1 VacA protein. Purified oligomeric toxins of each species were acid activated (a procedure which enhances cytotoxic activity) (14, 33) and then added to the neutral-pH medium overlying HeLa cells. In contrast to the wild-type VacA from strain 60190, the purified s2/m1 protein lacked detectable vacuolating activity as determined by both microscopic examination (data not shown) and analysis by a neutral-red uptake assay (Fig. 3). To verify that the lack of toxin activity was indeed a result of the presence of the s2 sequence rather than due to some other unrecognized mutation in a different region of *vacA*, we transformed *H. pylori* strain VM083 with plasmid pAV202 (containing the original s1 *vacA* sequence from wild-type *H. pylori* strain 60190), as described in Materials and Methods. PCR and sequence analysis confirmed that a double-crossover event had occurred in the bacterial chromosome, resulting in restoration of the original s1 *vacA* sequence. This strain, VM084, exhibited intact vacuolating activity (data not shown). Furthermore, introduction of an s1 sequence into the wild-type s2/m2 *vacA* allele of strain Tx30a resulted in expression of a chimeric s1/m2 VacA protein that exhibited vacuolating activity on Vero cells (data not shown), in contrast to the wild-type s2/m2 VacA from strain Tx30a, which lacked activity. These data confirm that the presence of a 12-amino-acid amino-terminal extension, consistently found in type s2 VacA proteins, confers a vacuolation-negative phenotype (1, 28).

Electrophysiologic properties of channels formed by wild-type and chimeric VacA toxins. Several recent studies have suggested that the vacuolating-cytotoxic effects of type s1 VacA are dependent on the capacity of the toxin to form anion-selective membrane channels (11, 53, 56, 57) and that a unique 32-amino-acid hydrophobic segment located at the amino terminus of type s1 VacA proteins plays an important role in membrane channel formation (32, 61). Consequently, we hypothesized that the hydrophilic s2 amino-terminal extension might interfere with the capacity of VacA to form membrane channels. To test this hypothesis, purified VacA preparations from *H. pylori* strains 60190 and VM083 were each incubated with planar lipid bilayers at pH 4. Type s1/m1 VacA from *H. pylori* strain 60190 induced a macroscopic current of 100 pA after incubation with bilayers for 51.5 ± 30.7 min, a result consistent with previous findings (24, 61). Addition to lipid bilayers of the s2/m1 toxin from strain VM083 resulted in a macroscopic current that was detectable only after a much longer delay than with the s1/m1 toxin under identical conditions (Table 2) ($P < 0.005$). Both of the VacA toxins examined formed channels with similar anion selectivities (Table 2). The currents formed by both toxins could be inhibited by 4,4′-diisothiocyanatostilbene-2, 2′-disulfonic acid, an inhibitor of anion transporters known to inhibit channels formed by VacA (2, 57) (data not shown). These data indicate that the presence of a type s2 12-amino-acid amino-terminal hydrophilic extension alters the capacity of VacA to form membrane channels.

We recently reported that a mutant s1/m1 toxin containing a deletion within the amino-terminal hydrophobic region, VacA-(Δ6–27), lacks detectable vacuolating activity and is capable of altering the channel-forming activity of wild-type s1/m1 toxin by a mechanism believed to involve the formation of heteromeric structures (61). Specifically, mixtures of wild-type s1/m1 VacA and VacA-(Δ6–27) formed channels in lipid bilayers significantly more slowly than did wild-type s1/m1 VacA alone, and channels formed by the mixture of toxins exhibited an altered anion selectivity (61). Therefore, we examined the elec-

TABLE 2. VacA channel-forming activity

VacA sample[a]	Time to 100 pA[b]	P_{Cl}/P_{Na}[c]
s1/m1	51.5 ± 30.7 (n = 12)	5.17 ± 0.89 (n = 11)
s2/m1	275 ± 120 (n = 12)	4.80 ± 0.93 (n = 10)
s1/m1 + s2/m1	30.1 ± 7.4 (n = 10)	5.35 ± 1.01 (n = 10)

[a] VacA toxins were purified from *H. pylori* strains 60190 (s1/m1) and VM083 (s2/m1). The toxins were acid activated and added to lipid bilayers at a concentration of 30 nM. In experiments using mixtures of different toxins (s1/m1 plus s2/m1), each toxin was present at a concentration of 30 nM.

[b] Time (in minutes) required to achieve a current of 100 pA. Acid-activated VacA was added to lipid bilayers in 100 mM sodium chloride, 5 mM citric acid, and 2 mM EDTA at pH 4.0, with an applied voltage of −50 mV. The results represent the means and standard deviations. The rate of macroscopic current formation was significantly higher for the s1/m1 VacA from wild-type strain 60190 than for the s2/m1 chimeric toxin (Student's t test; $P < 0.005$). Type s1/m1 VacA induced a detectable current at 30 min in 8 of the 12 experiments, whereas type s2/m1 VacA failed to induce current at this time point in any experiment. In contrast to s1/m1 VacA, the s2/m1 chimeric VacA toxin failed to induce a macroscopic current in five experiments (after periods of 210, 300, 360, 360, and 570 min) before the membrane ruptured. These results were excluded from our analysis.

[c] Permeability ratios were determined from the Goldman-Hodgkin-Katz equation (21) after measuring the membrane voltage for zero current (reversal potential) in asymmetric salt concentrations. Acid-activated VacA was added at a concentration of 30 nM. The *cis*-side buffer consisted of 200 mM sodium chloride, 5 mM citric acid, and 2 mM EDTA at pH 4.0; the *trans*-side buffer was identical except that it contained 100 mM sodium chloride. The results represent the means and standard deviations.

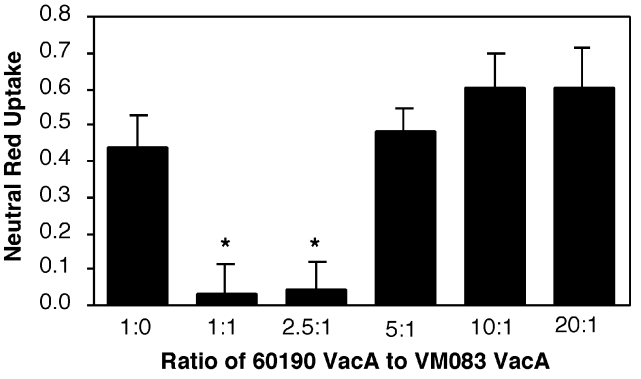

FIG. 4. Inhibition of type s1/m1 VacA activity by the type s2/m1 toxin from *H. pylori* strain VM083. Acid-activated s1/m1 VacA from *H. pylori* strain 60190 (5 μg/ml) was mixed with various concentrations of acid-activated s2/m1 VacA from *H. pylori* strain VM083 in the neutral-pH medium (MEM containing 10 mM ammonium chloride) overlying HeLa cells and incubated for 16 h at 37°C. Vacuolating activity was quantified using a neutral red uptake assay (9). The results represent the mean (± standard deviation) net absorbance at 540 nm from triplicate samples. The asterisks denote significant differences ($P < 0.05$) compared with wild-type s1/m1 VacA alone.

trophysiologic properties of channels formed by mixtures of s1/m1 VacA and the chimeric s2/m1 toxin. In contrast to the results we reported previously with mixtures of wild-type s1/m1 VacA and VacA-(Δ6–27), mixtures of s2/m1 VacA and s1/m1 toxin produced a macroscopic current at least as fast as the s1/m1 toxin alone, and the channels exhibited an anion selectivity that was indistinguishable from that of channels formed by s1/m1 VacA (Table 2). These results suggested that the s2/m1 and VacA-(Δ6–27) toxins interact differently with wild-type s1/m1 VacA or that perhaps s2/m1 VacA, in contrast to VacA-(Δ6–27), is unable to interact with wild-type s1/m1 VacA.

Dominant-negative phenotype of the type s2/m1 VacA toxin. It is widely believed that the cytotoxic activity of VacA is dependent upon the capacity of VacA to assemble into oligomeric structures (8, 11, 31, 33, 53, 61, 64). Therefore, we hypothesized that the nontoxic s2/m1 VacA protein might alter the cytotoxic activity of wild-type s1 VacA. Acid-activated wild-type s1/m1 VacA from strain 60190 was added to the neutral-pH culture medium overlying HeLa cells in the presence of various concentrations of acid-activated chimeric s2/m1 toxin from strain VM083. When the two proteins were present in equimolar concentrations, s2/m1 VacA completely inhibited the activity of the s1/m1 VacA from strain 60190 (Fig. 4). Significant inhibition was also detected when the ratio of s1/m1 to s2/m1 toxins was 2.5 to 1. An acidified buffer control lacked any inhibitory activity, and nonacidified s2/m1 VacA failed to inhibit the activity of the acid-activated s1/m1 toxin (data not shown). Thus, the acid-activated s2/m1 chimeric protein exhibited a dominant-negative phenotype. This dominant-interfering activity of s2/m1 VacA was similar to that described previously for VacA-(Δ6–27) (61).

Protein-protein interactions between different toxin species. The most likely mechanism by which VacA-(Δ6–27) and the chimeric s2/m1 VacA inhibit the activity of s1/m1 VacA is believed to involve the formation of heteromeric complexes with reduced activity (58, 61). However, the formation of heterooligomeric VacA complexes has not yet been demonstrated experimentally. To determine whether either VacA-(Δ6–27) or the chimeric s2/m1 VacA could physically interact with s1/m1 VacA, we needed to develop a means for distinguishing between different forms of VacA. To do this, we altered the type s1/m1 *vacA* allele in *H. pylori* strain 60190 as described in Materials and Methods so that the resulting strain, VT330, would express a modified toxin displaying a c-myc epitope in a location predicted to be surface exposed in the VacA oligomer (8, 55). This c-myc epitope-tagged form of VacA was secreted, formed oligomeric structures, and was indistinguishable from wild-type s1/m1 VacA with respect to HeLa cell-vacuolating activity [including being inhibited by both VacA-(Δ6–27) and s2/m1 VacA (data not shown)]. Moreover, the c-myc-tagged VacA, but not the wild-type s1/m1 VacA, was recognized in both enzyme-linked immunosorbent assays and immunoblot assays by a monoclonal anti-c-myc antibody and could be immunoprecipitated by the anti-c-myc antibody (data not shown). Thus, the c-myc-tagged VacA protein was functionally indistinguishable from wild-type s1/m1 VacA from *H. pylori* 60190, but the epitope-tagged VacA could be specifically recognized by the anti-c-myc antibody.

For our initial study of VacA protein-protein interactions, we selected c-myc-tagged VacA and VacA-(Δ6–27) for analysis. Because of the considerable difference in mass between the c-myc-tagged VacA and VacA-(Δ6–27) (Fig. 5, lanes a and b), the two toxins could be easily distinguished by immunoblotting using anti-VacA antisera. As expected, when incubated with either toxin alone, the anti-c-myc antibody immunoprecipitated c-myc-tagged VacA (Fig. 5, lanes c and e) but failed to immunoprecipitate VacA-(Δ6–27) (Fig. 5, lanes d and f). Similarly, when the anti-c-myc antibody was incubated with a non-acid-activated mixture of c-myc-tagged VacA and VacA-(Δ6–27), only the c-myc-tagged VacA was immunoprecipitated (Fig. 5, lane h). However, when mixtures of c-myc-tagged VacA and VacA-(Δ6–27) were acid activated (i.e., converted to monomers) and then neutralized to allow reannealing of the monomers (8, 34, 62), the anti-c-myc antibody immunoprecipitated both c-myc-tagged VacA and VacA-(Δ6–27) (Fig. 5, lane g). Thus, c-myc-tagged s1/m1 VacA and and VacA-(Δ6–27) could form heteromeric structures.

In order to detect interactions between the c-myc-tagged VacA and either the chimeric s2/m1 VacA or wild-type s1/m1 (which do not differ considerably in mass), various forms of purified VacA [wild-type s1/m1, s2/m1 VacA, and VacA-(Δ6–27)] were biotinylated as described in Materials and Methods. As expected, the anti-c-myc antibody failed to immunoprecipitate any of the acid-activated biotinylated toxins alone (Fig. 6, lane a). Similarly, the anti-c-myc antibody failed to immunoprecipitate any of the biotinylated VacA species from non-acid-activated mixtures of c-myc-tagged VacA and biotinylated VacA (Fig. 6, lane c). The biotinylated toxins were only immunoprecipitated by the anti-c-myc antibody from acid-activated mixtures of c-myc-tagged VacA and biotinylated VacA (Fig. 6, lane b). Based on these experiments, we conclude that s1/m1 VacA, s2/m1 VacA, and VacA-(Δ6–27) can each interact with c-myc-tagged s1/m1 VacA. The formation of heteromeric complexes only following acid activation of VacA species is consistent with the known capacity of s1/m1 VacA oligomers

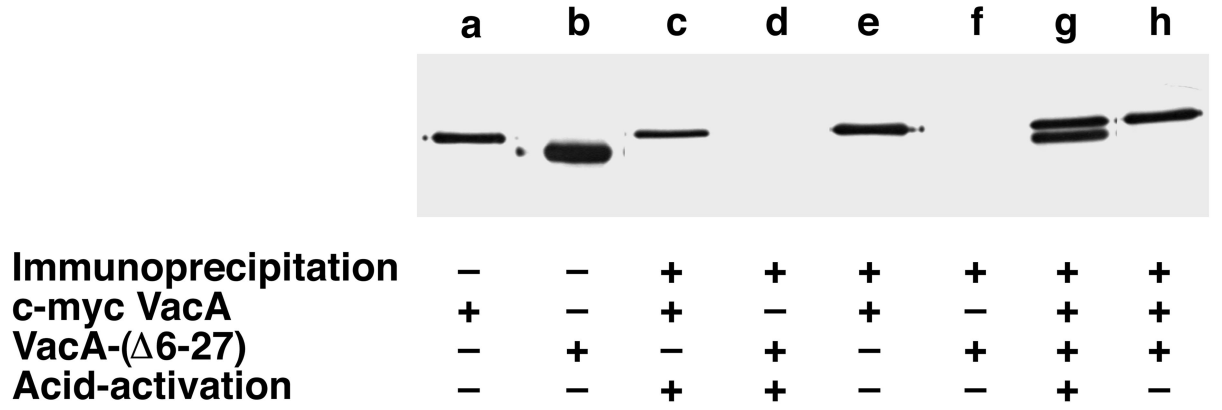

FIG. 5. Interactions of VacA-(Δ6–27) with c-myc-tagged s1/m1 VacA. c-myc-tagged VacA and nonbiotinylated VacA-(Δ6–27) could be readily distinguished by size differences in immunoblots using anti-VacA antiserum and an alkaline-phosphatase-conjugated secondary antibody (lanes a and b). As expected, the anti-c-myc antibody 9E10 immunoprecipitated c-myc-tagged VacA but not VacA-(Δ6–27) (lanes c to f). VacA-(Δ6–27) was successfully immunoprecipitated by the anti-c-myc antibody 9E10 if mixtures of c-myc-tagged VacA and VacA-(Δ6–27) were acid activated prior to the immunoprecipitation (lane g) but not in the absence of acid activation (lane h). +, present; −, absent.

to disassemble at acidic pH and subsequently reassemble into oligomeric structures upon neutralization (8, 34, 62).

DISCUSSION

There is considerable variation among *H. pylori* strains in production of vacuolating-toxin activity (1, 17, 30). This variation can be attributed in part to variation in VacA amino acid sequences (1, 17, 22, 51). Previous studies have provided evidence that sequence variation in the midregion of VacA (e.g., m1 versus m2), as well as sequence variation at the amino terminus of VacA (e.g., s1 versus s2), help to determine the level of toxic activity (1, 25, 28, 40). In the present study, we compared the actions of a wild-type s1/m1 toxin and a chimeric s2/m1 toxin. The amino acid sequences of these two toxins were identical, except that the latter toxin contained a type s2 segment in place of the original type s1 segment. One striking difference between the s2/m1 and s1/m1 toxins was that they underwent amino-terminal signal sequence cleavage at two different sites. Consequently, the mature secreted s2/m1 toxin contained a 12-amino-acid hydrophilic amino-terminal extension which was absent from the s1/m1 toxin. Our results confirm that the presence of this 12-amino-acid hydrophilic extension, characteristic of type s2 toxins, abolishes the capacity of VacA to induce cytoplasmic vacuolation in cultured cells (1, 28).

We hypothesized that the 12-amino-acid amino-terminal extension present in the type s2/m1 toxin might alter functions specified by the amino-terminal portion of the s1/m1 VacA protein. In particular, we reasoned that the s2 extension (being hydrophilic) might alter the conformation or mobility of a hydrophobic segment located at the amino terminus of type s1 VacA proteins. The amino-terminal type s1 VacA hydrophobic region is capable of inserting itself into membranes and dimerizing when expressed in *Escherichia coli* as part of a TOXCAT fusion protein (32, 46). Deletion of a portion of this region from s1/m1 VacA, as in VacA-(Δ6–27), results in a toxin that is defective in both vacuolating cytotoxic activity and the capacity to form membrane channels (61). Furthermore, various truncations or substitution mutations within this hydrophobic region ablate vacuolating-cytotoxin activity for HeLa cells when analyzed in a transient transfection system (12, 61, 63). When taken together, these previous results all indicate that the amino-terminal hydrophobic portion of type s1 VacA plays an important role in both the toxin's cytotoxic activity and its capacity to form membrane channels. Therefore, we hypothesized that the s1/m1 and s2/m1 VacA proteins might differ in the capacity to form membrane channels.

To test this possibility, we compared the capacities of the s1/m1 and s2/m1 VacA toxins to induce channels in planar lipid bilayers. Our results indicate that the chimeric s2/m1 toxin forms membrane channels at a rate significantly lower than that of the s1/m1 toxin. Once formed, the channels produced

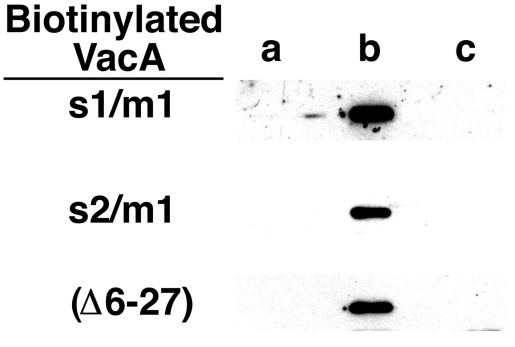

FIG. 6. Interactions of VacA-(Δ6–27), s1/m1 VacA, and chimeric s2/m1 VacA with c-myc-tagged s1/m1 VacA. Three species of purified VacA were biotinylated as described in Materials and Methods. Samples used for immunoprecipitations included acid-activated biotinylated VacA (lane a), acid-activated mixtures of biotinylated VacA and c-myc-tagged VacA (lane b), and untreated mixtures of biotinylated VacA and c-myc-tagged VacA (lane c). Immunoprecipitations using anti-c-myc antibody 9E10 were performed as described in Materials and Methods. Immunoprecipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose, and biotinylated VacA was detected with streptavidin-conjugated horseradish peroxidase and enhanced chemiluminescence. Biotinylated VacA species were successfully immunoprecipitated only if mixtures of biotinylated VacA and c-myc VacA were acid activated prior to the immunoprecipitation (lane b).

by wild-type s1/m1 VacA and the chimeric s2/m1 VacA exhibit similar anion selectivities. Thus, the presence of the type s2 amino-terminal segment inhibits efficient formation of membrane channels but does not alter the anion selectivity of the channels that do form. It seems likely that differences in the capacities of s1 and s2 toxins to form channels in planar lipid bilayers are relevant to the different capacities of these proteins to exert cytotoxic effects on eukaryotic cells. Interestingly, the defects in channel formation exhibited by s2/m1 VacA in the present study are not identical to the defects detected previously with VacA-(Δ6–27) (61). VacA-(Δ6–27) formed channels that were less anion selective than the channels formed by either wild-type s1/m1 VacA or s2/m1 VacA (61), and some preparations of VacA-(Δ6–27) lacked any detectable channel-forming activity (unpublished data). Presumably, a more drastic alteration in VacA-lipid interactions occurs due to the deletion of the amino-terminal hydrophobic region in VacA-(Δ6–27) than occurs due to the presence of the type s2 hydrophilic extension in s2/m1 VacA.

We reported previously that the mutant toxin VacA-(Δ6–27), when mixed in an equimolar ratio with wild-type s1/m1 VacA, inhibited the cytotoxic activity of the wild-type toxin (61). In the present study, we have identified a second form of VacA (s2/m1 VacA) that exhibits a similar dominant-negative phenotype. Thus, the addition of a hydrophilic amino-terminal extension in s2/m1 VacA and deletion of a large segment of an amino-terminal hydrophobic region in VacA-(Δ6–27) each altered VacA structure in ways that permitted the emergence of a dominant-negative phenotype. Presumably, an important characteristic of both of these dominant-negative mutant toxins is their retained capacity to assemble into oligomeric structures. In future studies, it will be important to carefully test other inactive mutant forms of VacA for the presence of a similar dominant-negative phenotype. Previous work using VacA inactivated by treatment with diethyl pyrocarbonate (61), as well as preliminary studies with additional inactive forms of VacA (containing deletions elsewhere in VacA, containing certain single-amino-acid substitutions, or belonging to the s2/m2 family), reveal that some inactive forms of VacA either fail to exhibit such a phenotype or possess an inhibitory effect considerably less prominent than that exhibited by VacA-(Δ6–27) or s2/m1 VacA (M. S. McClain and T. L. Cover, unpublished data). Further analysis will be required to decipher why there is variability among inactive forms of VacA in the potencies of dominant-negative effects.

A dominant-negative phenotype is most commonly observed when a nonfunctional mutant protein interferes with the proper assembly, folding, or function of oligomeric protein complexes (58). Therefore, we hypothesized that both s2/m1 VacA and VacA-(Δ6–27) are capable of interfering with the assembly or function of oligomeric structures containing wild-type s1/m1 VacA. In support of this hypothesis, experiments in the present study demonstrate for the first time that VacA-(Δ6–27) and s2/m1 VacA are indeed capable of interacting with s1/m1 VacA to form heterooligomeric complexes. Such heteromeric complexes formed only if the different VacA species were each acid activated and then shifted to neutral pH, i.e., conditions that promote disassembly of VacA oligomers followed by oligomer reassembly (8, 34, 62). Similarly, s2/m1 VacA and VacA-(Δ6–27) exhibited a dominant-negative phenotype in cell culture assays only if these proteins were first acid activated. This correlation supports a model in which the dominant-negative phenotype results from formation of heteromeric structures with defective activity and is consistent with the hypothesis that VacA oligomerization is required for cytotoxic activity.

Why heteromeric VacA complexes (containing both wild-type s1/m1 VacA and dominant-negative mutant toxins) might be inactive remains incompletely understood. We noted in a previous study that mixtures of VacA-(Δ6–27) and wild-type s1/m1 VacA formed channels less efficiently than did the wild-type s1/m1 VacA alone and that channels formed by such mixtures had an altered anion selectivity compared to wild-type s1/m1 VacA channels (61). Therefore, we speculated that alterations in VacA channel function might account for the dominant-negative phenotype of VacA-(Δ6–27) (61). In contrast, we have tested the capacities of mixtures of s2/m1 and s1/m1 VacA to form channels and have discovered that such mixtures form channels at least as efficiently as wild-type s1/m1 VacA alone. These results suggest that, although the process of VacA-induced cell vacuolation seems to be dependent on the formation of membrane channels (11, 24, 53, 56, 57), channel formation alone is not sufficient for VacA to induce cell vacuolation. Further work will be required to elucidate the precise mechanism by which s2/m1 VacA exerts its inhibitory action. Although it is possible that s2/m1 VacA somehow interferes with channel formation in a subtle manner that cannot be detected with the present lipid bilayer assays, it seems more likely that s2/m1 VacA blocks a step in the cellular intoxication process that is distinct from membrane channel formation. We speculate that VacA heterooligomers containing s2/m1 VacA might be defective in their intracellular trafficking and localization and thus might differ from s1/m1 homooligomers in their ability to induce vacuolation. Alternatively, heterooligomers containing s2/m1 VacA might be defective in interacting with an important but not-yet-identified intracellular target.

The presence of the 12-amino-acid "type s2" amino-terminal extension markedly alters the functional properties of type s2 toxins in two different in vitro assay systems (with endpoints of vacuolating cytotoxicity or membrane channel formation) compared with those of a type s1 toxin. Nevertheless, *H. pylori* strains encoding type s2 toxins are found commonly in human stomachs, and strains lacking *vacA* alleles or containing nonsense mutations in *vacA* seem to be quite rare. This suggests that, despite exhibiting apparent defects in in vitro assays, type s2 VacA toxins likely serve important functions in vivo which confer a selective advantage over *vacA*-null strains. In accordance with this hypothesis, Salama et al. (47) recently reported that an *H. pylori* strain (SS1) encoding a type s2/m2 VacA toxin (60) colonized a mouse model significantly more efficiently than did the isogenic *vacA*-null mutant strain.

We speculate that the ability of type s2 VacA proteins to form anion-selective membrane channels, even in the absence of cell-vacuolating activity, is an important function of VacA in vivo. For example, it has been suggested that such channels might promote the release of small metabolites, such as bicarbonate and pyruvate, from gastric epithelial cells in vivo, which might be favorable for growth of *H. pylori* in the gastric mucus layer (53). Analysis of the channel-forming properties of type

s2/m2 forms of VacA has not been conducted in any detail due to difficulties in purifying sufficient quantities of such toxins, but our preliminary investigations indicate that s2/m2 toxins can form anion-selective membrane channels with properties similar to those formed by the s2/m1 VacA protein analyzed in this study (data not shown). In addition to channel-forming activity, type s2 VacA proteins may also have other important biological functions in vivo, similar to those reported for type s1/m1 VacA toxins (13, 18, 36, 42, 43).

The dominant-negative phenotype of the s2/m1 VacA protein could also have important implications for growth of *H. pylori* in vivo. For example, since expression of certain forms of VacA seems to enhance the capacity of *H. pylori* to colonize the gastric mucosa in a mouse model (47), it seems possible that the capacity of *H. pylori* (expressing fully active forms of VacA) to colonize the stomach could be attenuated in the presence of dominant-negative forms of VacA. *H. pylori* strains expressing type s2/m1 forms of VacA occur quite rarely in human stomachs compared to strains expressing other forms of VacA (s1/m1, s1/m2, and s2/m2). Nevertheless, strains expressing s2/m1 forms of VacA have occasionally been isolated from human stomachs (29), and infection with multiple *H. pylori* strains is not uncommon (23, 26, 54). Based on the results of the present study, we predict that s2/m1 toxins will exhibit a dominant-negative phenotype in a mixed infection and might be capable of inhibiting colonization by *H. pylori* strains expressing type s1/m1 forms of VacA. Thus, the capacity of secreted proteins from one bacterium to alter the actions of proteins from other bacteria may represent an important form of bacterial competition. In support of the hypothesis that dominant-negative forms of bacterial proteins may have important actions in vivo, two recent reports indicated that administration of dominant-negative mutant forms of anthrax protective antigen were effective in blocking the activity of anthrax toxin in vivo (49, 50). The mechanism of these dominant-negative mutants of protective antigen is presumed to be similar to that proposed here for VacA, i.e., involving the formation of dysfunctional heterooligomeric structures. Thus, dominant-negative forms of secreted bacterial proteins not only may contribute to bacterial competition but also may eventually be used as a novel therapeutic approach for modifying the courses of various infectious diseases.

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