INFECTION AND IMMUNITY, June 2010, p. 2408–2417 Vol. 78, No. 6
0019-9567/10/$12.00 doi:10.1128/IAI.01194-09

Functional Characterization of the IlpA Protein of *Vibrio vulnificus* as an Adhesin and Its Role in Bacterial Pathogenesis▽

Kyung-Jo Lee,[1] Na Yeon Lee,[2] Yang-Soo Han,[1] Juri Kim,[2] Kyu-Ho Lee,[1] and Soon-Jung Park[2]*

Department of Environmental Science and Protein Research Center for Bio-Industry, Hankuk University of Foreign Studies, Yongin 449-791,[1] and Department of Environmental Medical Biology and Institute of Tropical Medicine, The Brain Korea 21 Project, Yonsei University College of Medicine, Seoul 120-752,[2] South Korea

Received 22 October 2009/Returned for modification 20 November 2009/Accepted 8 March 2010

***Vibrio vulnificus* is a Gram-negative bacterium that causes a fatal septicemia. One of its virulence factors is a membrane-bound lipoprotein, IlpA, which can induce cytokine production in human immune cells. In the present study, the role of IlpA as an adhesion molecule was investigated. An *ilpA*-deleted *V. vulnificus* mutant showed significantly decreased adherence to INT-407 human intestinal epithelial cells, which in turn resulted in reduced cytotoxicity. The Δ*ilpA* mutant recovered the adherence ability of the wild type by complementation in *trans* with the intact *ilpA* gene. In addition, pretreatment of *V. vulnificus* with anti-IlpA polyclonal antibodies resulted in a significant reduction of bacterial adherence. To localize the domain of IlpA required for cytoadherence, three truncated recombinant IlpA polypeptides were constructed and tested for the ability to adhere to human cells by a ligand-binding immunoblot assay and fluorescence microscopy. The polypeptide containing the carboxy (C)-terminal hydrophilic domain exhibited direct binding to INT-407 cells. Therefore, the C-terminal domain of IlpA allows this protein to be an adhesion molecule of *V. vulnificus*.**

Vibrio vulnificus is a Gram-negative pathogenic bacterium that is encapsulated, motile, and invasive. This pathogen is frequently associated with primary septicemia following the consumption of contaminated shellfish. Over 50% of patients with *V. vulnificus*-induced septicemia die due to multiorgan failure as a result of rapidly progressive shock syndrome (3, 19).

Diverse virulence factors have been proposed for *V. vulnificus*. Based on the attenuated mouse lethality of *V. vulnificus* mutants deficient in capsular polysaccharide (CPS) or exopolysaccharides (EPS), these molecules have been shown to be important for *V. vulnificus* pathogenesis (18, 38). Type IV pilin was confirmed to be involved in the virulence of *V. vulnificus* via genetic deletion of its structural gene, *pilA* (26, 27). In addition, motility was also discovered to be a critical virulence determinant of *V. vulnificus* (21). Secreted proteins, such as a cytolytic hemolysin (37) and an elastase (24), have been proposed to cause damage to host tissues, but *in vivo* studies using a knockout *V. vulnificus* mutant of *vvhA* (cytolysis gene) or *vvpE* (elastase gene) did not convincingly demonstrate that these proteins are key virulence factors causing lethality in mice or lysis of human cells (11, 30, 36).

The initial stage of microbial infection of host cells is mediated by interactions of the surface proteins of the pathogen with the connective tissues or epithelial cells of the host to facilitate bacterial adherence and/or to elicit signal transduction within host cells (5). For example, the most abundant outer membrane protein (OMP) of *V. vulnificus*, OmpU, has been identified to be involved in interactions with host extracellular matrix proteins, such as fibronectin (7). Knockout mutagenesis of the *ompU* gene results in the loss of cytoadherence as well as in decreased bacterial toxicity toward epithelial cells and mice. Membrane-bound IlpA lipoprotein was also identified to interact with host cells via stimulating the production of proinflammatory cytokines in human monocytes via Toll-like receptor 2 (TLR2). Lipid moieties attached to the N-terminal end of the processed IlpA protein are required for cytokine production (8). Subsequent investigation of *V. vulnificus* IlpA in this study showed that this protein is also important in bacterial adherence to human cell lines. Thus, we further identified the region of IlpA required for adherence to human cells.

MATERIALS AND METHODS

Strains, plasmids, and bacterial cultivation. The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown at 37°C in Luria-Bertani (LB) broth (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 1% [wt/vol] NaCl, pH 7.5) supplemented with ampicillin at 100 μg/ml for the maintenance of plasmids. Various strains of *V. vulnificus* were cultured in LBS broth (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 2% [wt/vol] NaCl, pH 7.5) at 30°C with tetracycline (2 μg/ml) when needed. All medium components were purchased from Difco, and the chemicals and antibiotics were obtained from Sigma.

Construction of a plasmid expressing a truncated form of IlpA lacking the C terminus. A plasmid, p*ilpA*[1-169], was constructed to express a mutated recombinant IlpA (rIlpA) polypeptide in which 99 amino acids at the C terminus were missing. The *ilpA′* DNA fragment of 729 bp, which encodes amino acid residues from the N terminus to the 169th amino acid of IlpA, was amplified from the genomic DNA of wild-type *V. vulnificus* by a PCR using the primers ilpA-comF (5′-GGTTGGATCCATTGGTGAGCT-3′; the underlined sequence denotes a BamHI restriction site) and ilpAΔ170-CR (5′-CCCAAGCTTTCAGTCACGA ACGGTCGCAAGTAG-3′; the underlined sequence indicates a HindIII restriction site), digested with BamHI and HindIII, and then cloned into the broad-host-range plasmid pLAFR5. The resultant plasmid, p*ilpA*[1-169], was transformed into *E. coli* SM10 λ*pir* and then transferred into the *V. vulnificus ilpA* mutant by conjugation. The exconjugants were selected on thiosulfate-citrate-bile-sucrose agar containing tetracycline (2 μg/ml). Expression of the truncated polypeptide in the Δ*ilpA* mutant was examined by Western blotting using anti-IlpA polyclonal antibodies.

Cell line adherence assay. Adherence assays were performed with the INT-407 cell line, which is derived from human intestinal epithelial cells (ATCC CCL-6), in 24-well culture plates. Each well of the culture plates was seeded with 2×10^5

* Corresponding author. Mailing address: Department of Environmental Medical Biology, Yonsei University College of Medicine, 134 Shinchon-dong, Seodaemun-gu, Seoul 120-752, South Korea. Phone: 82-2-2228-1843. Fax: 82-2-363-8676. E-mail: sjpark615@yuhs.ac.

▽ Published ahead of print on 22 March 2010.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli strains		
DH5α	*supE44* Δ*lacU169* (ϕ80d*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogen
SM10 λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pir*, OriT of RP4, Km^r; conjugational donor	31
BL21(DE3)	F^- *dcm ompT hsdS* (r_B^- m_B^-) *gal* λ(DE3)	Invitrogen
JM109	*endA1 recA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^-) *relA1 supE44* Δ(*lac-proAB*) [F′ *traD3 6proAB lacI*^{q}*Z*ΔM15]	Qiagen
V. vulnificus strains		
ATCC 29307	Clinical isolate	American Type Culture Collection
YS101	Δ*ilpA* mutant of ATCC 29307	8
Plasmids		
pLAFR5	RP4; derivative of pLAFR3 containing a double *cos* cassette; Tc^r	16
pLAFR-ilpA	pLAFR5 with 2,135-bp *V. vulnificus ilpA*; Tc^r	8
p*ilpA*[1-169]	pLAFR5 with 729-bp *V. vulnificus* 3′-truncated *ilpA*; Tc^r	This study
pET-28b	Expression vector for a histidine-tagged protein	Novagen
pET-IlpA	pET-28b with 810-bp *ilpA* gene	8
pQE-30	Expression vector for a histidine-tagged protein	Qiagen
pQE-IlpA#1	pQE-30 with 273-bp *ilpA′* gene (encoding G24 to I114)	This study
pQE-IlpA#2	pQE-30 with 333-bp *ilpA′* gene (encoding A115 to Y225)	This study
pQE-IlpA#3	pQE-30 with 303-bp *ilpA′* gene (encoding I170 to W269)	This study

INT-407 cells and incubated overnight at 37°C in the presence of 5% CO_2. After removal of the medium and two washes with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.3), 1 ml of serum-free Dulbecco's modified Eagle medium (DMEM; Gibco-BRL) was added to the INT-407 cells. Various *V. vulnificus* strains (ATCC 29307 harboring pLAFR5, YS101 carrying pLAFR5, YS101 carrying pLAFR-ilpA [8], and YS101 carrying p*ilpA*[1-169]) were grown at 30°C in LBS broth with 2 μg/ml tetracycline. At an optical density at 600 nm (OD_{600}) of 0.7, 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the bacterial cultures, and they were incubated for another 3 h at 30°C before being used for cytoadherence assays. Cell monolayers were inoculated in triplicate with 50 μl of the diluted bacteria to give a multiplicity of infection (MOI) of 10 and were incubated at 37°C in 5% CO_2 for 30 min. While *V. vulnificus* shows similar growth rates and maximum yields for growth at 30 and 37°C, INT-407 cells exhibit optimal growth at 37°C. Thus, the assays including both *V. vulnificus* and INT-407 cells were performed at 37°C. The monolayer was washed five times with prewarmed PBS to remove nonadherent bacteria. Following the last wash, the INT-407 cells were lysed with 0.1% Triton X-100 for 15 min. The bacteria were recovered from these cells with PBS, serially diluted 10-fold, and then plated on LBS agar.

Adherence-blocking experiments with anti-IlpA antibodies. Prior to cytoadherence assays, 1×10^8 cells of *V. vulnificus* were preincubated with anti-IlpA antibodies (8) for 1 h at various concentrations ranging from 10 to 30 μg/ml. Preimmune rat serum was used as a control immunoglobulin (IgG) to monitor the nonspecific effect of serum on the binding of *V. vulnificus* to INT-407 cells.

Cytotoxicity assays. The cytotoxicity of *V. vulnificus* toward INT-407 cells was measured using a CytoTox 96 nonradioactive cytotoxicity assay kit (Promega). This cytotoxicity kit measures the lactate dehydrogenase (LDH) activity released into the culture medium by lysed INT-407 cells. To measure the total LDH activity of the cell lines used in the assays, Triton X-100 was added to a final concentration of 1.0% (vol/vol) to lyse the host cells. Various *V. vulnificus* strains (ATCC 29307 harboring pLAFR5, YS101 carrying pLAFR5, YS101 carrying pLAFR-ilpA [8], and YS101 carrying p*ilpA*[1-169]) were grown at 30°C in LBS broth with 2 μg/ml tetracycline. At an OD_{600} of 0.7, the expression of IlpA protein was induced with 1 mM IPTG for 3 h. Monolayers of INT-407 cells were then inoculated in triplicate with 50 μl of the diluted bacteria to give an MOI of 10 or 50 and were incubated at 37°C in 5% CO_2 for 1 to 3 h. The released LDH activity was then determined by colorimetric assay according to the manufacturer's instructions and is presented as the percentage of LDH activity relative to the total LDH activity of the cells lysed by a treatment with 1.0% Triton X-100.

Preparation of human PBMCs and cytokine assays. Human peripheral blood mononuclear cells (PBMCs) were prepared by density gradient centrifugation, using Ficoll (Amersham Biosciences), and were resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Prepared PBMCs (1×10^5 cells per well) were cultured in 96-well plates and then incubated for 18 h with the lysates of various *V. vulnificus* strains, concanavalin A (ConA), or bovine serum albumin (BSA) at a concentration of 10 μg/ml. The levels of gamma interferon (IFN-γ) in the cell-free supernatants were determined using enzyme-linked immunosorbent assays (BD Biosciences). The concentration of IFN-γ was determined based on the standard curve for commercially available IFN-γ (BD Biosciences).

Expression and purification of rIlpA protein and its truncated polypeptides. The full-length IlpA protein was expressed in *E. coli* by use of an overexpression plasmid, pET-IlpA (8). A DNA fragment containing the open reading frame (ORF) of IlpA was dissected into three parts (Fig. 1). The 5′ region of *ilpA* (273 bp) was amplified from the genomic DNA of *V. vulnificus* ATCC 29307 by a PCR using the primers rIlpA#1FB (5′-CGACGGATCCATGAAATTTAGCCTTAA AGGTT-3′; the underlined region indicates a BamHI restriction site) and rIlpA#1RH (5′-CGCACTCGAGGTAGCCACGGTCTTCC-3′; the underlined region denotes a restriction site for HindIII), and the 3′ region of *ilpA* (303 bp) was amplified with another set of primers, rIlpA#3FB (5′-CGACGGATCCTC TATCAACCTAACTCCGG-3′; the underlined region indicates a restriction site

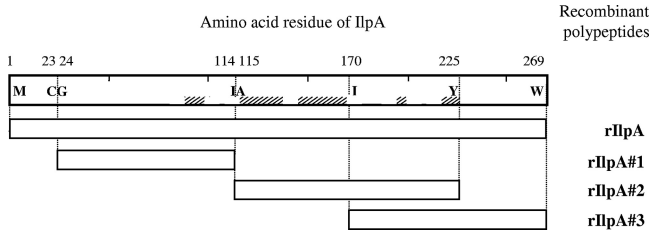


FIG. 1. rIlpA protein of *V. vulnificus* and its truncated derivatives used in this study. Four different rIlpA polypeptides were prepared to contain the complete IlpA ORF (rIlpA) and the N-terminal (rIlpA#1), central (rIlpA#2), and C-terminal (rIlpA#3) regions. Amino acid residues and their positions in each rIlpA polypeptide are displayed with alphabetical abbreviations and numbers, respectively. The hatched areas indicate hydrophobic regions of the protein.

for BamHI) and rIlpA#3RH (5′-CCTGCTCGAGTTTCAAATGTGACGACT GCC-3′; the underlined region indicates a HindIII restriction site). The central part of the *ilpA* gene, which codes for amino acid residues constituting the major hydrophobic regions of IlpA, was amplified as a 333-bp DNA fragment, using the following set of primers: rIlpA#2FB (5′-ATGCGGATCCGCGGCATTGGAT GACGG-3′; the underlined region indicates a BamHI site) and rIlpA#2RH (5′-CCTGCTCGAGCGAACGGTCGCAAGTAGG-3′; the underlined region denotes a HindIII site). The resultant *ilpA* DNA fragments were cloned into pQE30 (Qiagen) by using restriction sites for BamHI and HindIII to generate a series of overexpression plasmids for the truncated rIlpA polypeptides (pQE-IlpA#1, pQE-IlpA#2, and pQE-IlpA#3). These rIlpA polypeptides were expressed in a histidine-tagged form in *E. coli* JM109 cells to which 1 mM IPTG had been added and were purified using nickel-nitrilotriacetic acid affinity chromatography as described by the manufacturer (Qiagen).

Ligand-binding immunoblot assay. Each well of a 24-well culture plate was seeded with 2×10^5 INT-407 cells and incubated overnight at 37°C in the presence of 5% CO_2. After removal of the medium and two washes with DMEM, the cells were incubated in 1 ml of serum-free DMEM containing 10 μg of the rIlpA protein (8) as described by Jin et al. (12). After five washes with DMEM, the cells were disrupted with 70 μl of lysis buffer (10 mM Tris-HCl, pH 8.4, and 0.8% SDS) and boiled for 10 min. To examine whether rIlpA was stably bound to INT-407 cell monolayers, a portion of the lysate (200 μg of protein per well) was subjected to SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% nonfat dry milk in TBST (Tris-buffered saline with 0.1% Tween 20) for 1 h at room temperature, treated with anti-IlpA antibodies (8) at 4°C overnight, and subsequently incubated with alkaline phosphatase (AP)-conjugated anti-rat IgG (Sigma-Aldrich). To determine the domain of IlpA responsible for binding to host cells, the ligand-binding immunoblot assays were performed with the three truncated rIlpA polypeptides tagged with histidine residues, and then the bound polypeptide was detected using anti-poly-His monoclonal antibodies (Sigma). Immunoreactive bands were visualized by using a nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) system (Promega).

To monitor the protein levels of INT-407 cells after each treatment, the INT-407 cell lysates used for the above ligand-binding immunoblot assays were subjected to SDS-PAGE and then transferred to a PVDF membrane. The membranes were blocked with 5% nonfat dry milk in TBST for 1 h at room temperature, treated with anti-human Cu/Zn superoxide dismutase (Cu/Zn SOD) antibodies (Millipore) at 4°C overnight, subsequently incubated with AP-conjugated anti-rabbit IgG (Sigma-Aldrich), and then visualized using a kit containing NBT and BCIP (Promega).

Fluorescence microscopy. Intact rIlpA and three truncated rIlpA polypeptides, rIlpA#1, rIlpA#2, and rIlpA#3, were conjugated with a fluorescent probe by use of an Alexa Fluor 555 microscale protein labeling kit (A30007; Molecular Probes). Briefly, 100 μg of recombinant polypeptide was incubated with Alexa Fluor 555-succinimidyl ester, and then unincorporated probe was removed by spin filters supplied with the kit. The fluorescence of the proteins at 280 nm and at 555 nm was measured to ensure appropriate labeling of the recombinant polypeptides as directed by the manufacturer. INT-407 cells were seeded and grown on glass coverslips for 24 h. Cells were blocked with 1% BSA for 30 min in DMEM, followed by a 1 h-treatment with 10 μg/ml Alexa Fluor 555-labeled polypeptides at 37°C. Cells were washed with PBS three times and fixed in 3.7% paraformaldehyde-PBS at room temperature for 30 min. After being washed three times with PBS, the coverslips were then mounted on glass slides by use of a mounting medium that included an antifading agent and sodium azide (fluorescence mounting medium; Dako) and were then observed under a confocal laser scanning microscope (LSM700; Carl Zeiss MicroImaging). For an assay to examine the competition of binding to INT-407 cells by intact rIlpA and rIlpA#3, Alexa Fluor 555-labeled full-length rIlpA (10 μg/ml) was mixed with unlabeled rIlpA#3 at molar ratios of 1:1, 1:5, and 1:10 and then incubated with INT-407 cells as described above.

Statistical analyses. Results are expressed as means ± standard deviations for three independent experiments. Statistical analysis was performed using analysis of variance (ANOVA). Differences were judged as statistically significant when the P value was <0.05. Statistical analysis for pairwise comparisons was performed using Student's t test (SigmaPlot, version 9; Systat Software Inc.). Differences were considered significant if the P values were <0.05.

RESULTS

Role of IlpA protein in cytoadherence of *V. vulnificus*. *V. vulnificus* IlpA was isolated by immunoscreening of an expression library with antibodies raised against a fraction containing *V. vulnificus* OMPs, and it was further identified as an immunogenic protein of this microorganism (8). Some bacterial OMPs have been reported to have dual functions as adhesins and immunogens, such as the surface adhesion protein of *Streptococcus pneumoniae* (PsaA) (20) and the NadA adhesion protein of *Neisseria meningitidis* (6). Thus, in the present study, we examined in more detail the role of the IlpA protein in the interactions of *V. vulnificus* with host cells, particularly in the adherence of this organism to the human intestinal epithelial cell line INT-407 (Fig. 2A). When the ratio of bacteria to INT-407 cells was 10:1, the percentage of adhered wild-type *V. vulnificus* carrying pLAFR5, the vector plasmid, was about 10% of the bacteria initially added to the assay mixture. In the same assay with the Δ*ilpA* mutant YS101 carrying pLAFR5, the portion of bacterial cells adhered to INT-407 cells was significantly lowered, i.e., 2.1% of the added bacteria ($P = 0.0026$; Student's t test). However, when YS101 was carrying pLAFR-ilpA, an *lipA*$^+$-containing plasmid, cytoadherence of this strain recovered almost (9.7%) to that of the wild type carrying pLAFR5.

The role of the IlpA protein in cytoadherence of wild-type *V. vulnificus* was further confirmed by examining if masking of IlpA on the bacterial surface with anti-IlpA antibodies inhibited bacterial adherence (Fig. 2B). Wild-type *V. vulnificus* was treated for 30 min with various concentrations of anti-IlpA antibodies (ranging from 10 to 30 μg) prior to the adherence assay. As a control, wild-type *V. vulnificus* that had been incubated with rat preimmune serum at the same concentrations was used for the adherence tests. When wild-type *V. vulnificus* was preincubated with anti-IlpA antibodies, the levels of cytoadherence decreased gradually, in a dose-dependent manner, to 1.1%. Statistical analysis using ANOVA indicated a significant decrease in bacterial cytoadherence upon incubation with anti-IlpA antibodies ($P = 6.2E^{-08}$). In contrast, 7.4 to 9.0% of the added *V. vulnificus* bacteria adhered to the cells treated with preimmune serum, regardless of the concentration of IgG. ANOVA showed the absence of a meaningful difference in this set of data ($P = 0.75$). The blocking effect of anti-IlpA antibodies on bacterial adherence was not derived from agglutination of bacterial cells in the presence of anti-IlpA antibodies (data not shown).

Role of IlpA protein in cytotoxicity of *V. vulnificus* to INT-407 cells. We examined whether a deficiency of IlpA affects the ability of *V. vulnificus* to lyse INT-407 cells. After INT-407 cells were incubated with either the wild type carrying pLAFR5 or *V. vulnificus* Δ*ilpA* carrying pLAFR5 at two different MOIs, 10 and 50, the viability of the INT-407 cells was monitored for 3 h by measuring released LDH (Fig. 3). When INT-407 cells were treated with wild-type *V. vulnificus* carrying pLAFR5 at an MOI of 10, the percentages of released LDH increased gradually, to 95% lysis, as the incubation time extended to 3 h. When INT-407 cells were exposed to the larger number of wild-type *V. vulnificus* cells, i.e., an MOI of 50, they were completely lysed during 2 h of incubation. In contrast, cytotoxicity of the Δ*ilpA* mutant carrying pLAFR5 was estimated to be significantly lower than that of the wild type at both MOIs ($P < 0.01$; Student's t test), except for the 3-h incubation at an MOI of 50 (Fig. 3B), which was not different from the other incubations ($P = 0.59$; ANOVA).

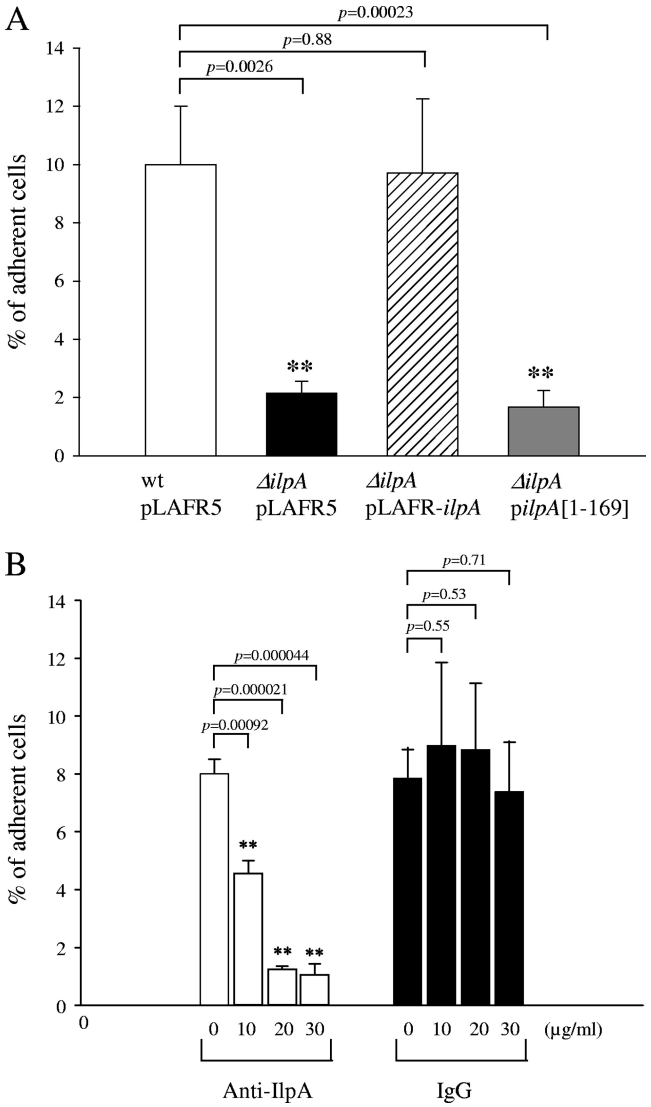


FIG. 2. Effects of *ilpA* mutation and exogenously added anti-IlpA antibodies on adherence of *V. vulnificus* to INT-407 cells. (A) Cytoadherence of various strains of *V. vulnificus* to INT-407 cells. *V. vulnificus* cells (wild-type *V. vulnificus* ATCC 29307 carrying pLAFR5 [white bar], Δ*ilpA* mutant YS101 carrying pLAFR5 [black bar], YS101 carrying a complementation plasmid, pLAFR-ilpA [hatched bar], and YS101 carrying p*ilpA*[1-169] [gray bar]) were added to INT-407 cells at an MOI of 10 and incubated for 30 min. The percentage of bacterial cells adhered to INT-407 cells was determined by measuring the CFU of the retrieved bacteria. Adherence assays with various *V. vulnificus* strains and INT-407 cells were performed in triplicate, and data are presented as averages with standard deviations. Two asterisks indicate binding levels that were significantly ($P < 0.01$; Student's *t* test) different from that of wild-type *V. vulnificus* carrying pLAFR5. ANOVA on this set of data indicated a statistically significant difference ($P = 0.00027$). (B) Inhibition of cytoadherence of wild-type *V. vulnificus* to INT-407 cells by exogenous addition of anti-IlpA antibodies. Prior to the cytoadherence test, wild-type *V. vulnificus* was treated for 30 min with various concentrations of anti-rIlpA antibodies (10, 20, and 30 μg/ml) (white bars) or with rat preimmune serum (black bars). The percentage of bacterial cells that adhered to INT-407 cells was determined as described above. Two asterisks indicate binding levels that were significantly different from that of untreated wild-type *V. vulnificus* ($P < 0.01$; Student's *t* test). Data using anti-IlpA demonstrated a significant difference ($P = 6.2E^{-08}$; ANOVA), whereas data with IgG did not indicate any significant difference ($P = 0.75$; ANOVA).

In subsequent experiments, we examined whether a loss of cytotoxicity in the Δ*ilpA* mutant could be recovered by introducing a complementation plasmid, pLAFR-ilpA. Inclusion of a complementation plasmid in the Δ*ilpA* mutant increased LDH activity up to that of the wild type carrying the vector plasmid ($P < 0.05$; Student's *t* test).

Direct binding of rIlpA to INT-407 cells. We then investigated whether the IlpA protein binds directly to host cells. INT-407 cells were incubated with rIlpA, washed, and then prepared as lysates. Western blot analysis of these lysates with anti-rIlpA antibodies clearly showed that the rIlpA protein was stably bound to INT-407 cells (Fig. 4A). As a loading control, the amount of human Cu/Zn SOD in the INT-407 cell lysates used for this assay was also monitored (17). Figure 4B shows a similar level of human Cu/Zn SOD in each sample, suggesting that the absence of immunoreactive bands in Fig. 4A, lane 2, was not due to a difference in the amounts of human cell lysates loaded on PAGE gels.

Localization of the cytoadherence domain of the IlpA protein. An *in silico* analysis of the amino acid sequence of IlpA revealed major hydrophobic regions, which may be localized within the membrane and connect the extracellular, lipidated N-terminal region and the putatively extracellular C-terminal region (Fig. 1) (http://www.expasy.ch/tools/protscale.html). Therefore, the whole *ilpA* gene was divided into three parts, as shown in Fig. 1, and cloned into an expression vector; the N-terminal hydrophilic, central hydrophobic, and C-terminal hydrophilic regions of IlpA were made as truncated and histidine-tagged forms and were named rIlpA#1, rIlpA#2, and rIlpA#3, respectively. Truncated rIlpA polypeptides were examined for the ability to specifically adhere to INT-407 cells (Fig. 5A). Only the rIlpA#3 polypeptide, containing the C-terminal domain, was clearly observed in INT-407 cell lysates. In contrast, the other two rIlpA polypeptides, rIlpA#1 and rIlpA#2, were not detected in INT-407 cell lysates. In the same manner, the amount of human Cu/Zn SOD in the INT-407 cell lysates was detected as a loading control for each sample (Fig. 5B) and indicated that similar amounts of human cell lysates were used in this study.

Binding of truncated rIlpA polypeptides to INT-407 cells was directly visualized using a fluorescence microscope (Fig. 6A). Three truncated rIlpA polypeptides were labeled by a red fluorescent probe, Alexa Fluor 555, and fluorescent recombinant polypeptides were then added to INT-407 cells. Only the rIlpA#3-treated cells demonstrated fluorescence on the host cells, whereas INT-407 cells incubated with the other two rIlpA polypeptides did not show any fluorescence signal.

Full-length rIlpA protein was labeled with a fluorescent probe and used for the binding experiments. The results demonstrated that the intact IlpA protein binds to INT-407 cells, as shown in the binding assay with rIlpA#3 (Fig. 6B, panel a). To confirm the specificity of IlpA binding to INT-407 cells, the binding reaction mixture containing fluorescent rIlpA protein and INT-407 cells was examined with various amounts of unlabeled rIlpA#3 added, from 10 to 100 μg (Fig. 6B, panels c, e, and g). Fluorescent signals on INT-407 cells gradually disappeared as the added amount of rIlpA#3 increased. Addition of unlabeled rIlpA#1 or rIlpA#2 did not affect the degree of rIlpA binding to INT-407 cells (data not shown). These two independent experiments clearly demonstrated that IlpA di-

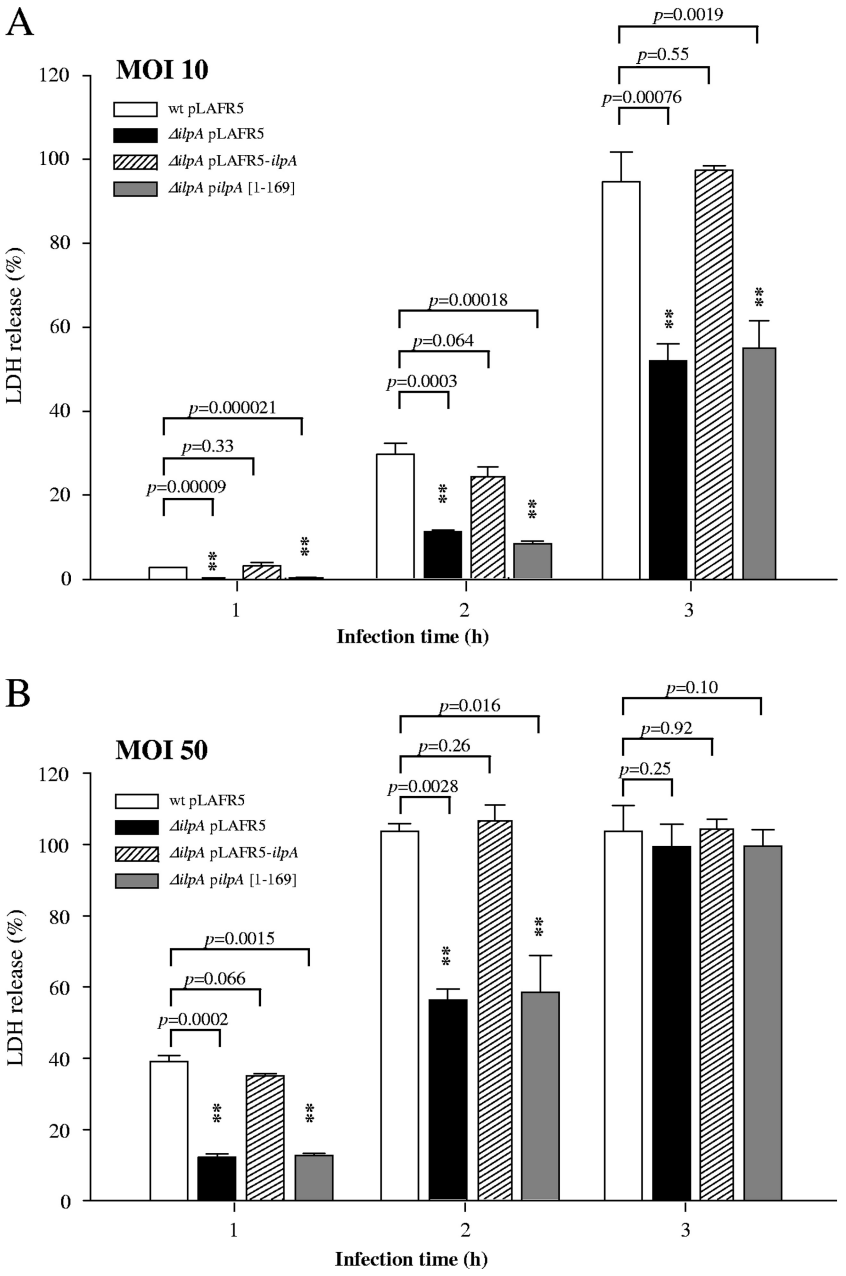


FIG. 3. Effect of *ilpA* mutation on cytotoxicity of *V. vulnificus* to INT-407 cells. Using a CytoTox96 assay kit, the LDH activity released from lysed INT-407 cells was measured after exposure of cells to various *V. vulnificus* strains (wild-type *V. vulnificus* ATCC 29307 carrying pLAFR5 [white bar], Δ*ilpA* mutant YS101 carrying pLAFR5 [black bar], YS101 carrying a complementation plasmid, pLAFR-ilpA [hatched bar], and YS101 carrying p*ilpA*[1-169] [gray bar]). INT-407 cells (1×10^5) were incubated with *V. vulnificus* for various times (1, 2, and 3 h) at MOIs of 10 (A) and 50 (B). Data are means with standard deviations for three independent experiments. Two asterisks indicate LDH activity that was significantly different ($P < 0.01$; Student's *t* test) from that of the wild type carrying pLAFR5. ANOVA on bacterial cytotoxicity for 1, 2, and 3 h of incubation at an MOI of 10 indicated statistically significant differences ($P = 1.1E^{-05}$, $1.4E^{-06}$, and $5.0E^{-05}$, respectively). Cytotoxicity data for 1 and 2 h of incubation at an MOI of 50 also demonstrated significant differences ($P = 1.1E^{-09}$ and $6.2E^{-06}$, respectively), whereas 3 h of incubation at an MOI of 50 did not show any difference by ANOVA ($P = 0.59$).

rectly binds to the surfaces of INT-407 cells via its C-terminal region, which functions as an adhesin domain.

Role of the C-terminal region of IlpA in pathogenesis of *V. vulnificus*. In a previous report (8), the Δ*ilpA* mutant was demonstrated to be attenuated in virulence. This mutant stimulated less cytokine production from monocytes due to the absence of the lipidated N terminus of IlpA. In the present study, it was also shown that the C-terminal region of IlpA plays an important role in cytoadherence. To dissect the factor causing virulence attenuation of the Δ*ilpA* mutant, the phenotypes of the Δ*ilpA* mutant carrying p*ilpA*[1-169], in which a C-terminally truncated IlpA polypeptide was produced, were analyzed (data not shown). This strain showed a similar level of adherence of INT-407 cells (1.7%) to that shown by the Δ*ilpA* mu-

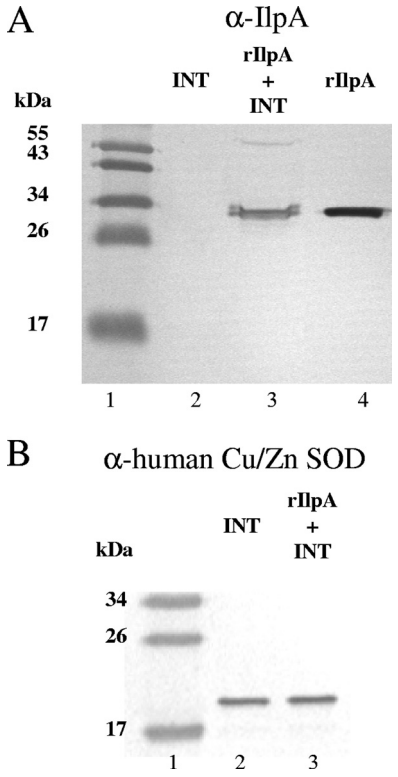


FIG. 4. Determination of binding of rIlpA to INT-407 cells by a ligand-binding immunoblot assay. (A) Binding of rIlpA to INT-407 cells. INT-407 cells (2×10^5) were incubated with 10 μg rIlpA, and the cell lysate was analyzed to see if INT-407 cells were bound by added bacterial proteins via Western blotting using anti-IlpA antibodies. (B) Loading control. The same INT-407 cell lysates were also analyzed for their contents of Cu/Zn SOD via Western blotting using specific antibodies, as described in Materials and Methods. Lanes 1, protein size marker; lanes 2, INT-407 cells alone; lanes 3, INT-407 cells with rIlpA; lane 4, rIlpA alone (0.1 μg).

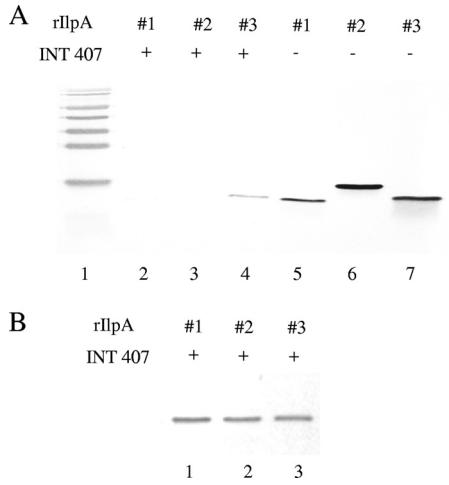


FIG. 5. Determination of binding region of IlpA via ligand-binding immunoblot assay, using truncated rIlpAs and INT-407 cells. INT-407 cells (2×10^5) were incubated with 10 μg of three truncated IlpA polypeptides, rIlpA#1, rIlpA#2, and rIlpA#3, as shown in Fig. 1. (A) Cell lysates were then analyzed to see if they were bound by added polypeptides via Western blotting using anti-His antibodies. Lane 1, protein size marker; lane 2, INT-407 cells with rIlpA#1; lane 3, INT-407 cells with rIlpA#2; lane 4, INT-407 cells with rIlpA#3; lane 5, rIlpA#1 alone (0.1 μg); lane 6, rIlpA#2 alone (0.1 μg); lane 7, rIlpA#3 alone (0.1 μg). (B) As a loading control, the same INT-407 cell lysates were also analyzed for their Cu/Zn SOD content via Western blotting using specific antibodies, as described in Materials and Methods.

tant carrying pLAFR5 (2.1%) (Fig. 2A). This result confirmed the important role of the C-terminal region of IlpA in cytoadherence, as shown in Fig. 5 and 6.

Next, we examined if the C-terminal region is also involved in stimulation of proinflammatory cytokine production in human PBMCs. When human PBMCs were treated with a lysate of the wild type carrying pLAFR5, the secretion of IFN-γ by PBMCs was 93 pg/ml (Fig. 7). In contrast, treatment of PBMCs with a lysate of the Δ*ilpA* mutant carrying pLAFR5 resulted in a significantly lowered level of IFN-γ production (4 pg/ml) ($P = 0.044$; Student's *t* test), which was comparable to those of the control cells, such as PBMCs treated with medium only or with BSA. When a lysate of the Δ*ilpA* mutant carrying pLAFR-ilpA was used to challenge PBMCs, IFN-γ production was restored to the level induced by wild-type *V. vulnificus* lysate (69 pg/ml) ($P = 0.47$; Student's *t* test). Lysates of the Δ*ilpA* mutant carrying p*ilpA*[1-169] were effective at inducing cytokine production by human PBMCs (56 pg/ml), showing similar levels to those shown by the wild type carrying the vector and the Δ*ilpA* mutant carrying pLAFR-ilpA ($P = 0.32$ and 0.38, respectively). Human PBMCs treated with ConA, which served as a positive control for this experiment, showed IFN-γ production at 79 pg/ml. The capacity of the amino-terminal domain to reproduce most of the immunostimulating activity of IlpA suggests that the C-terminal domain of IlpA may not have a major role in immunostimulating activity of IlpA.

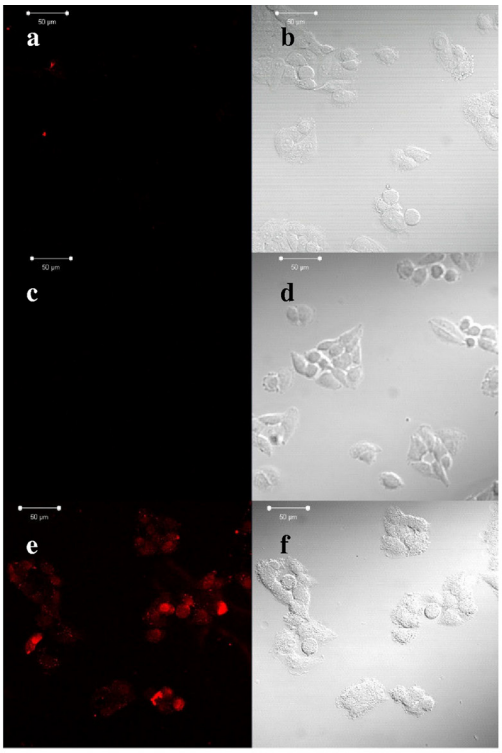
Since the plasmid encoding the C-terminally deleted IlpA, p*ilpA*[1-169], was found to be defective in recovery of cytoadherence of the Δ*ilpA* mutant (Fig. 2A) but efficient in recovery of immunostimulation of the Δ*ilpA* mutant (Fig. 7), we further examined whether this truncated IlpA was involved in bacterial cytotoxicity to INT-407 cells. The Δ*ilpA* mutant carrying p*ilpA*[1-169] exhibited lowered levels of cytotoxicity, 27 to 57% of those of the wild type carrying the vector plasmid, except for the 3-h incubation at an MOI of 50 (Fig. 3). Therefore, all of the results for the Δ*ilpA* mutant carrying p*ilpA*[1-169] suggest that decreased cytotoxicity of the Δ*ilpA* mutant was caused mainly from reduced cytoadherence.

DISCUSSION

The severe and rapid cytopathological characteristics of *V. vulnificus* infection have led to this organism being considered a model system with which to investigate host-pathogen interactions. Among various virulence-related phenotypes, the ability of bacterial cells to adhere to host cells via bacterial adhesins is critical for the initial pathogenic interaction. The significant decrease in cytoadherence of a *V. vulnificus* Δ*ilpA* mutant to INT-407 human cells suggests a potential role of the IlpA protein as an adhesin for this pathogenic microorganism.

The role of IlpA in adherence of *V. vulnificus* to human intestinal epithelial cells, such as INT-407 cells, was clearly demonstrated by comparative cytoadherence assays of wild-

A



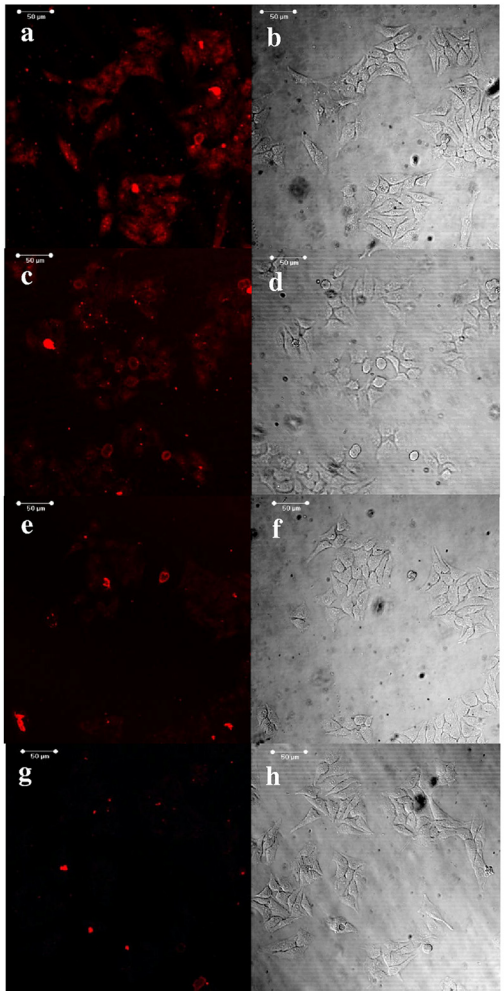
rIlpA#1

rIlpA#2

rIlpA#3

B

Labeled rIlpA : Unlabeled rIlpA#3



(1:0)

(1:1)

(1:5)

(1:10)

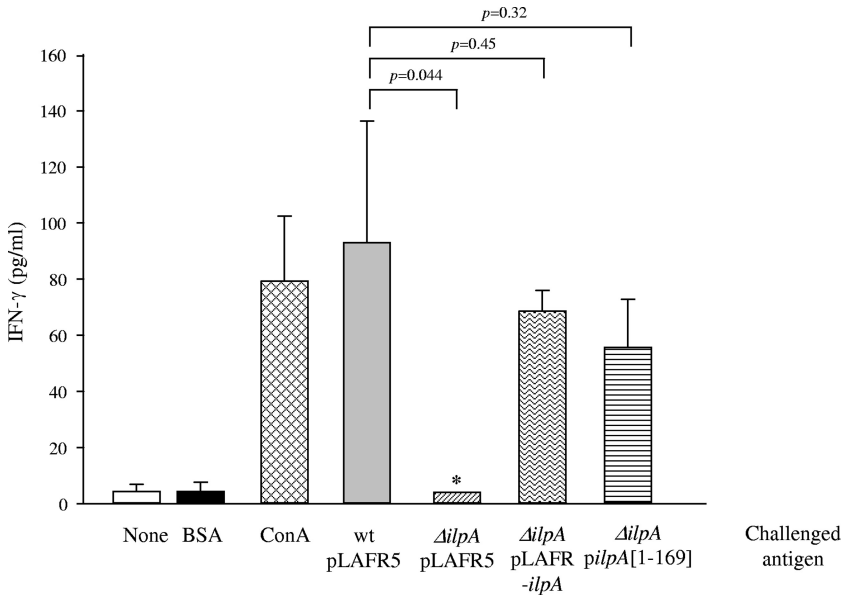


FIG. 7. Cytokine production in human PBMCs induced by *V. vulnificus* cell lysates. PBMCs (1×10^5) prepared from human blood were incubated for 18 h with lysates (10 μg/ml) of bacterial strains (wild-type *V. vulnificus* ATCC 29307 carrying pLAFR5, the Δ*ilpA* mutant YS101 carrying pLAFR5, YS101 carrying pLAFR-ilpA, and YS101 carrying p*ilpA*[1-169]). The concentrations of IFN-γ stimulated by lysates, medium (none), BSA, and ConA were determined using a human IFN-γ enzyme-linked immunosorbent assay kit (BD Biosciences). Error bars represent the standard deviations for three independent experiments. IFN-γ production induced by each lysate of *V. vulnificus* was measured in triplicate for each experiment. Data with an asterisk indicate that the IFN-γ level was significantly different ($P < 0.05$; Student's *t* test) from that of the wild type carrying pLAFR5. ANOVA on this set of data indicated a significant difference ($P = 0.00087$).

type and Δ*ilpA* mutant *V. vulnificus* (Fig. 2A). In addition, a ligand-binding immunoblot assay using rIlpA (Fig. 4A) and microscopic observation using a fluorescent rIlpA (Fig. 6B) demonstrated an association of rIlpA with INT-407 cells. As a result of reduced adherence, the mutant exhibited significantly less cytotoxicity than the wild type (Fig. 3).

IlpA has previously been reported to be an immunogenic protein of *V. vulnificus* which stimulates cytokine production in human blood cells (8). The *V. vulnificus* IlpA protein is presumed to be modified by acylation of its first cysteine residue with fatty acid chains after its N-terminal leader peptide of 22 amino acid residues is processed. Its lipid moiety is essential to activate the TLR2-mediated signaling pathway and then to trigger cytokine production by human monocytes. Therefore, the IlpA protein may play important roles in *V. vulnificus* pathogenicity via at least dual functions during the initial interactions of bacteria with host cells, i.e., cytoadherence to intestinal epithelial cells and immunostimulation in monocytes.

Some membrane-bound lipoproteins have been reported to have multiple functions. One of the best-characterized adhesin/immunogen proteins is the 33-kDa PsaA protein of *S. pneumoniae*, which directly binds to the target host cells, such as nasopharyngeal epithelial cells (9, 23, 29). Mutation in the *psaA* gene caused pleiotropic effects on the growth, oxidative stress response, adherence, and virulence of *S. pneumoniae* (14). PsaA was also found to be one of the main pneumococcal antigens and causes a distinct immune response in a mouse model which is different from those mediated by other pneumococcal antigens (25). Another group of well-known bacterial adhesins are pili or fimbriae (28). The thin-curled fimbriae of *Salmonella enterica* and the fimbrillin of *Porphyromonas gingivalis* are able to trigger cytokine production from human cells via the action of TLR2 (10, 33). Therefore, many membrane-bound lipoproteins as well as surface proteins of diverse bacteria play multiple roles, and the IlpA protein of *V. vulnificus* may be added to the list of bacterial proteins performing at least two distinct roles, facilitating cytoadherence and eliciting cytokine production.

Both an *in silico* analysis of the amino acid sequence of IlpA and a comparison with the structure of the IlpA homolog lipoprotein 9 of *Staphylococcus aureus* (35) allowed us to lo-

FIG. 6. INT-407 cells incubated with rIlpA polypeptides labeled by a fluorescent dye. (A) Binding of truncated rIlpAs to INT-407 cells. INT-407 cells (5×10^4) incubated with 10 μg of Alexa Fluor 555-labeled rIlpA#1 (a and b), rIlpA#2 (c and d), or rIlpA#3 (e and f) were observed under a laser scanning confocal microscope. The images were collected as serial sections at 0.5-μm intervals and analyzed by Zeiss LSM image browser software (ZEN2009LE; Zeiss). Fluorescent images are presented on the left (a, c, and e), and light micrographs are presented on the right (b, d, and f). Bars, 50 μm. (B) Binding of intact rIlpA to INT-407 cells. INT-407 cells (5×10^4) incubated with 10 μg of Alexa Fluor 555-labeled full-length rIlpA (a and b) were observed as described above. In addition, the same binding assays were preformed in the presence of 10 (c and d), 50 (e and f), or 100 μg unlabeled rIlpA#3. Fluorescent images are presented on the left (a, c, e, and g), and light micrographs are presented on the right (b, d, f, and h). Bars, 50 μm.

calize the major hydrophobic regions that might reside in the outer leaflet of the membrane and connect two extracellular domains, a lipidated N-terminal region and a putatively extracellular C-terminal region (data not shown). Ligand-binding immunoblot assays using three different truncated rIlpAs (Fig. 5A) showed that the polypeptide rIlpA#3, including the C-terminal one-third of the IlpA protein, was as efficient in binding to INT-407 cells as the intact IlpA protein (Fig. 4A). The direct binding of rIlpA#3 was confirmed by microscopic observation using fluorescent probe-labeled truncated rIlpAs (Fig. 6). On the other hand, two other truncated rIlpA polypeptides, rIlpA#1 and rIlpA#2, did not show any binding to host cells. Therefore, the extracellular domains of IlpA may be accessible to interaction with the host cell surface, and some of the secondary structures in the extracellularly exposed rIlpA#3 possibly participate in binding to the specific host receptor, which has not yet been identified.

Using recombinant PsaA protein covalently bound to fluorescent spheres, E-cadherin was identified as a host cellular receptor for PsaA of *S. pneumoniae* (1). A *Campylobacter jejuni* adhesin, JlpA lipoprotein, was found to trigger inflammatory/innate immune responses in host cells during *C. jejuni* infection via direct interaction with surface-exposed heat shock protein 90α and subsequent activation of NF-κB and p38 mitogen-activated protein (MAP) kinase in host cells (13). A family of opacity outer membrane proteins (Opa proteins) plays a significant role during cytoadherence of *Neisseria gonorrhoeae* to human cells (20). Their interaction with host cells is mediated by several cellular components, including heparin sulfate proteoglycan, extracellular matrix components, and CD66 family receptors (4). Interaction between Opa and these host components activates a signaling pathway resulting in transcellular transversal of this pathogen across human cells (34).

In addition to IlpA, two adhesins have been documented for *V. vulnificus*, including OmpU (7) and PilA (26). Further investigation using *V. vulnificus* strains deficient in multiple loci of these adhesin genes will be needed to evaluate the contribution of each adhesin to overall cytoadherence of *V. vulnificus* to diverse cell types. Due to a lack of this information, it is premature to suggest that each adhesin functions with a specific cell type or broad range of cell types. Human epidermoid carcinoma (HEp-2) cells were used for studies of OmpU (7) and PilA (26), whereas the INT-407 cell line, derived from human intestinal epithelial cells, has been used for IlpA. However, the effects of *ilpA* mutation on adherence and cytotoxicity are not confined to the specific host cell line used in this study, since the same effects were also observed in similar experiments using HEp-2 cells (Y.-S. Han, S.-J. Park, and K.-H. Lee, unpublished data). Fibronectin, especially its RGD repeat, was found to be a receptor for the OmpU protein in *V. vulnificus* (7) as well as in *Vibrio cholerae* (32). However, no information is available on a receptor for *V. vulnificus* PilA.

Among the three adhesins of *V. vulnificus*, only IlpA has been shown to stimulate the production of proinflammatory cytokines in human monocytes. However, the possibility that the other adhesins of *V. vulnificus* have immunostimulating activity cannot be excluded. A *V. cholerae ompU* mutant was defective in activation of a proinflammatory response (2), and the *V. vulnificus* outer membrane protein fraction, including OmpU, was effective at eliciting immunization of mice against *V. vulnificus* (15). In addition, a *Pseudomonas aeruginosa pilA* mutant induced less secretion of cytokines and showed a lack of neutrophil chemotaxis in a murine model (22).

As mentioned above, IlpA stimulates cytokine production from human blood cells, and TLR2 of the host cells is a critical component for this process (8). A direct association between IlpA and TLR2 has not been proven and needs to be verified by further investigation. Similarly, further study is required to identify the host receptor molecule(s) for the adhesion domain of IlpA and to elucidate subsequent signaling processes in host cells. Therefore, we are currently screening for a host receptor(s) for *V. vulnificus* IlpA. The study of adhesin-receptor interactions will elucidate the complex interactive networks of diverse adhesins of *V. vulnificus* during the initial pathogenicity process, in which each adhesin might interact with unique or common receptor molecules to efficiently connect the required steps for successful cytoadherence to and entry into specific cell types.

ACKNOWLEDGMENTS

This study was supported by the Basic Science Research Program through the National Research Foundation (NRF), funded by the Ministry of Education, Science and Technology (grant 2009-0070681 to K.-H.L.), and partly by the Gyeonggi Regional Research Center (GRRC) program (Protein Research Center for Bio-Industry) of Gyeonggi Province, Republic of Korea.

REFERENCES

1. **Anderton, J. M., G. Rajam, S. Romero-Steiner, S. Summer, A. P. Kowalczyk, G. M. Carlone, J. S. Sampson, and E. W. Ades.** 2007. E-cadherin is a receptor for the common protein pneumococcal surface adhesin A (PsaA) of *Streptococcus pneumoniae*. Microb. Pathog. **42:**225–236.
2. **Bandyopadhaya, A., S. Bhowmick, and K. Chaudhuri.** 2009. Activation of proinflammatory response in human intestinal epithelial cells following *Vibrio cholerae* infection through PI3K/Akt pathway. Can. J. Microbiol. **55:** 1310–1318.
3. **Blake, P. A., M. H. Merson, R. E. Weaver, D. G. Hollis, and P. C. Heublin.** 1979. Disease caused by a marine vibrio: clinical characteristics and epidemiology. N. Engl. J. Med. **300:**1–5.
4. **Dehio, C., E. Freissler, C. Lanz, O. G. Gomez-Duarte, G. David, and T. F. Meyer.** 1998. Ligation of cell surface heparin sulfate proteoglycans by antibody-coated beads stimulates phagocyte uptake into epithelial cells: a model for cellular invasion by *Neisseria gonorrhoeae*. Exp. Cell Res. **242:**528–539.
5. **Di Martino, P., J. Rebiere-Huet, and C. Hulen.** 2000. Effects of antibiotics on adherence of *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* to A549 pneumocyte cells. Chemotherapy **46:**129–134.
6. **Franzoso, S., C. Mazzon, M. Sztukowska, P. Cecchini, T. Kasin, B. Capecchi, R. Tavano, and E. Papini.** 2008. Human monocytes/macrophages are a target of *Neisseria meningitidis* adhesin A (NadA). J. Leukoc. Biol. **82:**1100–1110.
7. **Goo, S. Y., J.-H. Lee, W. H. Kim, K.-L. Han, D.-K. Park, H.-J. Lee, S. M. Kim, K.-S. Kim, K.-H. Lee, and S.-J. Park.** 2006. Identification of OmpU of *Vibrio vulnificus* as a fibronectin-binding protein and its role in bacterial pathogenesis. Infect. Immun. **74:**5586–5594.
8. **Goo, S. Y., Y. S. Han, W. H. Kim, K.-H. Lee, and S.-J. Park.** 2007. *Vibrio vulnificus* IlpA-induced cytokine production is mediated by Toll-like receptor 2. J. Biol. Chem. **282:**27647–27658.
9. **Gor, D. O., X. Ding, Q. Li, J. R. Schreiber, M. Dubinsky, and N. S. Greenspan.** 2002. Enhanced immunogenicity of pneumococcal surface adhesin A by genetic fusion to cytokines: an evaluation of protective immunity in mice. Infect. Immun. **70:**5589–5595.
10. **Hajishengallis, G., M. Martin, H. T. Sojar, A. Sharma, R. E. Schifferle, E. DeNardin, M. W. Russell, and R. J. Genco.** 2002. Dependence of bacterial protein adhesins on Toll-like receptors for proinflammatory cytokine induction. Clin. Diagn. Lab. Immunol. **9:**403–411.
11. **Jeong, K. C., H. S. Jeong, J. H. Rhee, S. E. Lee, S. S. Chung, A. M. Starks, G. M. Escudero, P. A. Gulig, and S. H. Choi.** 2000. Construction and phenotypic evaluation of a *Vibrio vulnificus vvpE* mutant for elastolytic protease. Infect. Immun. **68:**5096–5106.
12. **Jin, S., A. Joe, J. Lynett, E. Hani, P. Sherman, and V. L. Chan.** 2001. JlpA, a novel surface-exposed lipoprotein specific to *Campylobacter jejuni*, mediates adherence to host epithelial cells. Mol. Microbiol. **39:**1225–1236.
13. **Jin, S., Y. C. Song, A. Emili, P. M. Sherman, and V. L. Chan.** 2003. JlpA of *Campylobacter jejuni* interacts with surface-exposed heat shock protein 90α

and trigger signalling pathways leading to activation of NF-κB and p38 MAP kinase in epithelial cells. Cell. Microbiol. **5:**165–174.
14. **Johnston, J. W., L. E. Myers, M. M. Ochs, W. H. Benjamin, Jr., D. E. Briles, and S. K. Hollingshead.** 2004. Lipoprotein PsaA in virulence of *Streptococcus pneumoniae*: surface accessibility and role in protection from superoxide. Infect. Immun. **72:**5858–5867.
15. **Jung, C., M. Park, and M. Heo.** 2005. Immunization of major outer membrane protein of *Vibrio vulnificus* elicits protective antibodies in a murine model. J. Microbiol. **43:**437–442.
16. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger.** 1988. Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. Gene **70:**191–197.
17. **Keller, G. A., T. G. Warner, K. S. Steimer, and R. A. Hallewell.** 1991. Cu,Zn superoxide dismutase is a peroxisomal enzyme in human fibroblasts and hepatoma cells. Proc. Nat. Acad. Sci. U. S. A. **88:**7381–7385.
18. **Kim, H.-S., S.-J. Park, and K.-H. Lee.** 2009. Role of NtrC-regulated exopolysaccharides in the biofilm formation and pathogenic interaction of *Vibrio vulnificus*. Mol. Microbiol. **74:**436–453.
19. **Klontz, K. C., S. Lieb, M. Schreiber, H. T. Janowski, L. M. Baldy, and R. A. Gunn.** 1988. Syndromes of *Vibrio vulnificus* infections. Clinical and epidemiologic features in Florida cases, 1981–1987. Ann. Intern. Med. **109:**318–323.
20. **Kupsch, E.-M., B. Knepper, T. Kuroki, I. Heuer, and T. F. Meyer.** 1992. Variable opacity (Opa) outer membrane proteins account for the cell tropisms displayed by *Neisseria gonorrhoeae* for human leukocytes and epithelial cells. EMBO J. **12:**641–650.
21. **Lee, J. H., J. B. Rho, K. J. Park, C. B. Kim, Y. S. Han, S. H. Choi, K.-H. Lee, and S.-J. Park.** 2004. Role of flagellum and motility in pathogenesis of *Vibrio vulnificus*. Infect. Immun. **79:**4905–4910.
22. **Lorenz, E., D. C. Chemotti, K. Vandal, and P. A. Tessier.** 2004. Toll-like receptor 2 represses nonpilus adhesin-induced signaling in acute infections with the *Pseudomonas aeruginosa pilA* mutant. Infect. Immun. **72:**4561–4569.
23. **Miyaji, E. N., W. O. Dias, M. Gamberini, V. C. B. C. Gabara, R. P. F. Schenkman, J. Wild, P. Riedl, J. Reimann, R. Schirmbeck, and L. C. C. Leite.** 2002. PsaA (pneumococcal surface adhesin A) and PspA (pneumococcal surface protein A) DNA vaccines induce humoral and cellular immune responses against *Streptococcus pneumoniae*. Vaccine **20:**805–812.
24. **Miyoshi, S., and S. Shinoda.** 2000. Microbial metalloproteases and pathogenesis. Microbes Infect. **2:**91–98.
25. **Palaniappan, R., S. Singh, U. P. Singh, S. K. K. Sakthivel, E. W. Ades, D. E. Briles, S. K. Hollingshead, J. C. Paton, J. S. Sampson, and J. W. Lillard, Jr.** 2005. Differential PsaA-, PspA-, PspC-, and PdB-specific immune responses in a mouse model of pneumococcal carriage. Infect. Immun. **73:**1006–1013.
26. **Paranjpye, R. N., and M. S. Strom.** 2005. A *Vibrio vulnificus* type IV pilin contributes to biofilm formation, adherence to epithelial cells, and virulence. Infect. Immun. **73:**1411–1422.
27. **Paranjpye, R. N., J. C. Lara, J. C. Pepe, C. M. Pepe, and M. S. Strom.** 1998. The type IV leader peptidase/N-methyltransferase of *Vibrio vulnificus* controls factors required for adherence to HEp-2 cells and virulence in iron-overloaded mice. Infect. Immun. **16:**5659–5668.
28. **Pizarro-Cerda, J., and P. Cossart.** 2006. Bacterial adhesion and entry into host cells. Cell **124:**715–727.
29. **Romero-Steiner, S., J. Caba, G. Rajam, T. Langley, A. Floyd, S. E. Johnson, J. S. Sampson, G. M. Carlone, and E. W. Ades.** 2006. Adherence of recombinant pneumococcal surface adhesin A (rPsaA)-coated particles to human nasopharyngeal epithelial cells for the evaluation of anti-PsaA functional antibodies. Vaccine **24:**3224–3231.
30. **Shao, C. P., and L. I. Hor.** 2000. Metalloprotease is not essential for *Vibrio vulnificus* virulence in mice. Infect. Immun. **68:**3569–3573.
31. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. BioTechnology **1:**784–791.
32. **Sperandio, V., J. A. Giron, W. D. Silveira, and J. B. Kaper.** 1995. The OmpU outer membrane protein, a potential adherence factor of *Vibrio cholerae*. Infect. Immun. **63:**4433–4438.
33. **Tukel, C., M. Raffatellu, A. D. Humphries, R. P. Wilson, H. L. Ndrews-Polymenis, T. Gull, J. F. Figueiredo, M. H. Wong, K. S. Michelsen, M. Akcelik, L. G. Adams, and A. J. Baumler.** 2005. CsgA is a pathogen-associated molecular pattern of *Salmonella enterica* serotype Typhimurium that is recognized by Toll-like receptor 2. Mol. Microbiol. **58:**289–304.
34. **Wang, J., S. D. Gray-Owen, A. Knorre, T. F. Meyer, and C. Dehio.** 1998. Opa binding to cellular CD66 receptors mediates the transcellular transversal of *Neisseria gonorrhoeae* across polarized T84 epithelial cell monolayers. Mol. Microbiol. **30:**657–671.
35. **Williams, W. A., R. Zhang, M. Zhou, G. Joachimiak, P. Gornicki, D. Missiakas, and A. Joachimiak.** 2004. The membrane-associated lipoprotein-9 GmpC from *Staphylococcus aureus* binds to the dipeptide GlycMet via side chain interactions. Biochemistry **43:**16193–16202.
36. **Wright, A. C., and J. G. Morris, Jr.** 1991. The extracellular cytolysin of *Vibrio vulnificus*: inactivation and relationship to virulence in mice. Infect. Immun. **59:**192–197.
37. **Wright, A. C., J. G. Morris, Jr., D. R. Maneval, Jr., K. Richardson, and J. B. Kaper.** 1985. Cloning of the cytotoxin-hemolysin gene of *Vibrio vulnificus*. Infect. Immun. **50:**922–924.
38. **Wright, A. C., L. M. Simpson, J. D. Oliver, and J. G. Morris, Jr.** 1990. Phenotypic evaluation of acapsular transposon mutants of *Vibrio vulnificus*. Infect. Immun. **58:**1769–1773.

Editor: S. R. Blanke