



A mammalian insulysin homolog is regulated by enzyme IIA^{Glc} of the glucose transport system in *Vibrio vulnificus*

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ABSTRACT

***Vibrio vulnificus* is an opportunistic human pathogen that causes severe infections in susceptible individuals. While the components of the *Escherichia coli* phosphoenolpyruvate: sugar phosphotransferase system (PTS) have been shown to regulate numerous targets, little such information is available for the *V. vulnificus* PTS. Here we show that enzyme IIA^{Glc} of the PTS regulates the peptidase activity of a mammalian insulysin homolog in *V. vulnificus*. While interaction of IIA^{Glc} with the insulysin homolog is independent of the phosphorylation state of IIA^{Glc}, only unphosphorylated IIA^{Glc} activates the insulysin homolog. Taken together, our results suggest that the *V. vulnificus* insulysin–IIA^{Glc} complex plays a role in survival in the host by sensing glucose.**

Structured summary:

MINT-8045996:

IIA *glu* (uniprotkb:Q7MBY2) binds (MI:0407) to vIDE (uniprotkb:Q7MIS6) by pull down (MI:0096)

MINT-8045817, MINT-8045967:

IIA *glu* (uniprotkb:Q7MBY2) physically interacts (MI:0915) with vIDE (uniprotkb:Q7MIS6) by pull down (MI:0096)

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1. Introduction

Vibrio vulnificus is a Gram-negative bacterium that is found in estuarine waters and frequently contaminates seafood. Strains of *V. vulnificus* are classified into three biotypes based on their biochemical characteristics and those belonging to biotype 1 are

responsible for the majority of human infections [1]. Biotype 2 strains are primary eel pathogens, while the biotype 3 appears to be emerged by recombination between biotypes 1 and 2 genomes and causes human wound infection [2]. Primary septicemia is the most lethal infection caused by *V. vulnificus*, with an average mortality rate exceeding 50%. Predisposing factors for severe *V. vulnificus* infection include chronic liver disease, elevated serum iron levels, low gastric acid, immunosuppression, and diabetes [3–5]. Most septic patients die of fulminant septicemia within 48 h after infection, implying that *V. vulnificus* cells subvert host innate immune responses upon infection.

The bacterial phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) participates in several physiological processes, in addition to its role in coupling phosphorylation to translocation of numerous sugars across the cytoplasmic membrane [6]. The PTS is composed of two general proteins, enzyme I (EI) and histidine-containing phosphocarrier protein (HPr), and a set of sugar-specific proteins collectively known as enzymes II. EI and HPr mediate phosphoryl transfer from PEP to enzyme II, which finally phosphorylates PTS sugar substrates during uptake. Therefore, the ratio of the phosphorylated forms of the PTS components increases in

Abbreviations: PEP, phosphoenolpyruvate; PTS, phosphoenolpyruvate:sugar phosphotransferase system; EI, enzyme I of the PTS; HPr, histidine-containing phosphocarrier protein; IIA^{Glc}, glucose-specific enzyme IIA of the PTS; TFA, trifluoroacetic acid; HPLC, high performance liquid chromatography; vIDE, *Vibrio vulnificus* insulin-degrading enzyme; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; NEM, N-ethylmaleimide; TPEN, N,N,N',N'-tetraakis(2-pyridylmethyl)ethylenediamine; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; PBS, phosphate-buffered saline; TCBS agar, thiosulfate citrate bile salts sucrose agar

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the absence and decreases in the presence of a PTS sugar. Roles of the PTS have been most extensively studied in *Escherichia coli*, and these roles include regulation of chemotaxis by EI [6], regulation of glycogen phosphorylase by HPr [7], and sequestration of the global repressor Mlc by glucose-specific enzyme IICB to induce expression of the PTS proteins in the presence of PTS sugars [8–11]. In addition, glucose-specific enzyme IIA (IIA^{Glc}) plays multiple roles such as regulation of adenyl cyclase, inducer exclusion of non-PTS sugars to preferentially consume glucose, inhibition of glycerol kinase activity, and regulation of fermentation/respiration switch protein [6,12,13].

Although the PTS has never been documented in *V. vulnificus*, we postulated that it might play important roles in *V. vulnificus* physiology. We therefore set out to isolate a protein(s) that interacts with and is regulated by IIA^{Glc} in *V. vulnificus* because all of the regulatory roles of the PTS reported to date are mediated through the direct interaction of the PTS components with their target proteins, and because IIA^{Glc} is the key regulatory component among Gram-negative bacterial PTSs [6,12–14].

2. Materials and methods

2.1. Strains, plasmids and culture media

Bacterial strains and plasmids used in this study are listed in Table 1. *V. vulnificus* strains were cultured in Luria-Bertani medium containing 2.5% NaCl (LBS) and all *E. coli* strains except BL21(DE3) derivatives were grown in M9 medium supplemented with 2% casamino acids and 1% glycerol. BL21 (DE3) strains were grown in LB medium.

To construct a *V. vulnificus* strain deleted for the mammalian insulysin homolog (hereafter referred as vIDE, which stands for *V. vulnificus* insulin-degrading enzyme), the region covering the gene (hereafter referred as *ideV*) equivalent to the locus *VV1_1977* in the sequenced *V. vulnificus* CMCP6 genome was amplified from the chromosomal DNA of *V. vulnificus* MO6-24/O [15] by PCR with primers 5'-AAACGCCACGGGGGCGCGCAGCATCTG-3' (an engineered *Apal* site underlined) and 5'-TTGCACTACTTGAGCTCAGTAGAGAAAATG-3' (an engineered *SacI* site underlined). This

fragment was ligated into pGEM-T Easy (Promega). The internal region of the *ideV* ORF was digested with *AflIII* and *XhoI* and replaced with the *neo* gene. This plasmid containing the kanamycin cassette within the *ideV* ORF was digested with *Apal* and *SacI* and cloned into the pDM4 vector [16] to make pDM4-vIDE. The *V. vulnificus* strain deleted for *ideV* was generated by homologous recombination using *E. coli* SM10 λ pir harboring pDM4-vIDE as a conjugal donor to *V. vulnificus* MO6-24/O [17].

To construct expression vectors for the vIDE, the coding region was amplified from the chromosomal DNA of *V. vulnificus* MO6-24/O using PCR with the primers 5'-TTTCGGAGACGCCATATGCACTT-AAGTCCA-3' (an engineered *NdeI* site underlined) and 5'-TTGCTTCTTTGTCTGACTGCTGTTTCTTTG-3' (an engineered *Sall* site underlined). After digestion, the *NdeI*-*Sall* fragment was inserted into the corresponding sites of pRE-His-Tag [18] to make pRH-vIDE for overexpression of the vIDE with an N-terminal His₆ and into pRE1 [19] to make pRE-vIDE for overexpression of the vIDE without His tags. To construct the expression vector pRH-vCrr for *V. vulnificus* IIA^{Glc} with His₆ at the N-terminus (His-IIA^{Glc}), the *crr* gene (equivalent to the locus *VV1_0212* in the CMCP6 strain) was amplified using PCR with the primers 5'-ACCTTAGGAGCATGACCATATGGGTCTGTT-3' (*NdeI* site underlined) and 5'-GGCAGTGCAATTGGATCCCTATCTTAGGTT-3' (*BamHI* site underlined). The PCR product was digested with *NdeI* and *BamHI* and cloned into pRE-His-Tag. The overexpression vector pRH-PtsI for *E. coli* EI with an N-terminal His₆ tag was constructed by moving the *NdeI*-*BamHI* fragment including the ORF for EI from pPR6 [20] into the corresponding sites of pRE-His-Tag.

To construct the expression vector for human insulysin, endogenous *NdeI* and *BamHI* sites in the plasmid containing the full-length human insulysin cDNA (ImaGenes) were removed by PCR using silent mutagenic primers. The DNA fragment extending from the 42nd ATG codon to the stop codon and containing a His₆ tag at the N-terminus was amplified using PCR with primers: forward primer, 5'-CTTACAGCCATATGCACCACCACCACCACCAATAATCCAGCCATC AAGAGAATAGGAAATCAC (*NdeI* site underlined); and reverse primer, 5'-GCATGGGGGATCCTCAGAGTTTTCAGCCATG (*BamHI* site underlined). After digestion, the *NdeI*-*BamHI* fragment was inserted into the corresponding sites of pET3a (Novagen).

Table 1
Bacterial strains and plasmids used in this study.

Strains or plasmids	Genotypes and/or descriptions	Source or reference
Bacterial strains		
<i>V. vulnificus</i> strains		
MO6-24/O	Wild-type, Biotype 1, Clinical isolate	[37]
MO6-24/O Δ ideV	MO6-24/O Δ ideV::Km ^r	This study
<i>E. coli</i> strains		
GI698	F ⁻ λ -lacI ^q lacPL8 ampC::P _{trp} cI	[21]
GI698 Δ pts	GI698 Δ (ptsH, ptsI, crr), Km ^r	[21]
BL21(DE3) pLysS		Novagen
SM10 λ pir	thi-1 thr leu tonA lacY supE recA::Rp4-2-Tc::Mu λ pir;Km ^r	[38]
Plasmids		
pRE1	Expression vector under control of λ P _L promoter, Ap ^r	[19]
pRE-His-Tag	pRE1-based expression vector for N-terminal His ₆ -tagged proteins, Ap ^r	[18]
pRH-vIDE	<i>ideV</i> cloned into pRE-His-Tag for expression of His ₆ -tagged vIDE, Ap ^r	This study
pRE-vIDE	<i>ideV</i> cloned into pRE1 for overexpression of vIDE, Ap ^r	This study
pRH-vCrr	<i>V. vulnificus</i> <i>crr</i> encoding IIA ^{Glc} cloned into pRE-His-Tag, Ap ^r	This study
pRH-PtsI	<i>E. coli</i> <i>ptsI</i> encoding EI cloned into pRE-His-Tag, Ap ^r	This study
pCR-BluntII-TOPO-hIDE	Cloning vector of human insulysin (hIDE), Km ^r	ImaGenes
pET3a	Expression vector for N-terminal His ₆ -tagged proteins under control of T7 promoter, Ap ^r	Novagen
pET-hIDE	hIDE-encoding gene cloned into pET3a	This study
pGEM-T Easy	Cloning vector for PCR product, Ap ^r	Promega
pDM4	Suicide vector for homologous recombination into <i>V. vulnificus</i> chromosome, Cm ^r	[16]
pDM4-vIDE	pDM4-based suicide vector for replacement of <i>ideV</i> with <i>neo</i> , Cm ^r and Km ^r	This study

Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Cm^r, chloramphenicol resistance.

2.2. Protein purification

All the proteins with N-terminal His₆ tags used in this study were overexpressed in *E. coli* Gl698Δpts strains [21] for pRE1-based vectors or BL21 (DE3) strains for pET vectors, and purified using TALON™ metal affinity resin (Clontech) according to the manufacturer's instructions. The proteins were eluted with 200 mM imidazole and further purified by gel filtration chromatography (Superose 12, 1.6 × 50 cm; GE Healthcare) in buffer A (20 mM HEPES–KOH, pH 8.0, containing 100 mM NaCl). The vIDE without a His₆ tag was overexpressed in *E. coli* strain Gl698Δpts harboring pRE-vIDE. Cells were harvested 20 h after induction with 1 mM tryptophan, disrupted by sonication and centrifuged at 100,000×g for 40 min at 4 °C. The vIDE was purified from the supernatant by DEAE-Sepharose (Sigma), HiLoad 16/60 Superdex75 prepgrade (GE Healthcare), and Mono Q 10/100 (GE Healthcare) chromatography according to the method described for purification of *E. coli* FrsA [13]. *E. coli* EI and HPr were purified as described previously [9]. Protein concentration was determined by bicinchoninic acid protein assay (Pierce).

2.3. Identification of a protein interacting with IIA^{Glc}

V. vulnificus MO6-24/O cells grown at 30 °C overnight in LBS (500 ml) were harvested, washed and resuspended in 10 ml of buffer A. Cells were disrupted by sonication and centrifuged at 100,000×g for 40 min at 4 °C. The supernatant (2 ml) was mixed with 2 mg of either IIA^{Glc} or His–IIA^{Glc}, and this mixture was incubated with 500 μl of TALON™ metal-affinity resin at 4 °C for 30 min. After incubation, the resin was harvested and washed with 1 ml of buffer A three times, and the proteins bound to the column were eluted with 500 μl of buffer A containing 200 mM imidazole. Aliquots of the eluted protein samples were analyzed by SDS–PAGE using a 4–20% gradient gel and staining with Coomassie blue. The protein band that specifically bound to His–IIA^{Glc} was excised from the gel and in-gel digestion and peptide mapping of tryptic digests were carried out using the Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems Inc.) as previously described [22,23].

To test the specificity of the interaction between IIA^{Glc} and the vIDE, the *E. coli* Gl698Δpts strain [21] harboring pRE-vIDE was grown in 50 ml of M9 medium, induced for protein expression with 1 mM tryptophan, harvested, washed, and resuspended in Buffer A. After purification, the vIDE (20 μg) was mixed with various concentrations of purified His–IIA^{Glc} (or His-tagged *E. coli* EI as a control) and 50 μl of TALON™ metal affinity resin in a total volume of 500 μl. After the mixture was loaded into a column, the column was washed and the proteins bound to the column were eluted with 50 μl of 2× SDS sample buffer and analyzed by 14% SDS–PAGE. Proteins were stained with Coomassie Brilliant Blue R and scanned with an LAS-3000 (Fujifilm) for densitometric analysis.

To test the phosphorylation dependence of the interaction between IIA^{Glc} and the vIDE, vIDE (20 μg) was incubated with EI (3 μg), HPr (4.5 μg) and His–IIA^{Glc} (10 μg) in a total volume of 500 μl of buffer A containing 2 mM DTT and 2 mM MgCl₂ in the presence of 1 mM glucose (to ensure complete dephosphorylation of His–IIA^{Glc}) or PEP (to ensure complete phosphorylation of His–IIA^{Glc}) and subjected to pull-down assays as described above.

2.4. Measurement of peptidase activity

Purified vIDE (1 μM) was incubated with human insulin (purchased from Sigma-Aldrich) in 100 mM Tris–HCl (pH 7.5) in the presence or absence of IIA^{Glc} (2 μM) at 30 °C. The reaction was terminated by adding SDS–PAGE loading buffer or 1/10 volume of 5.5% trifluoroacetic acid (Fluka). The degradation of insulin was mea-

sured by SDS–PAGE followed by staining with Coomassie Brilliant Blue R or injecting digests through high performance liquid chromatography (HPLC). For the HPLC analyses, samples were applied to C18 Hypersil™ Gold (Thermo) which had been washed with 0.1% trifluoroacetic acid in acetonitrile (B) and equilibrated with 0.1% trifluoroacetic acid in water (A). The insulin peak was resolved using a linear gradient of 0–60% B over 50 min, followed by 60–100% B over 10 min. A flow rate of 1.0 ml/min was used, and insulin was detected with a UV detector set at 215 nm.

2.5. Comparison of survival rate and virulence of *V. vulnificus* strains in mice

Wild-type *V. vulnificus* MO6-24/O and its otherwise isogenic vIDE deletion mutant strains were grown in LBS to OD₆₀₀ = 0.6. After wash with phosphate-buffered saline (PBS), the bacterial cells were resuspended in PBS at a concentration of 3 × 10⁶ cells/ml. Equal numbers of the two strains (3 × 10⁵ cells per each strain) were intraperitoneally injected into specific pathogen-free seven-week-old female ICR mice. The livers and spleens of the infected mice were removed 2 h after injection and homogenized in 5 ml of PBS. The homogenates were serially diluted in PBS and aliquots were spread onto the thiosulfate citrate bile salts sucrose (TCBS) agar plates in the presence or absence of kanamycin (250 μg/ml). The fraction of mutant cells was estimated by enumerating kanamycin-resistant colonies.

Seven-week-old female ICR mice were subcutaneously injected with iron dextran (80 μg/g body weight). After incubation for 1 h, 100 μl of freshly grown bacterial suspension in PBS (containing about 1.5 × 10⁴ of wild-type or vIDE deletion mutant *V. vulnificus* cells) was injected intraperitoneally into each mouse and the number of dead mice was determined.

All animal procedures were previously approved by the Institute of Laboratory Animal Resources at Seoul National University.

3. Results

3.1. An insulysin homolog interacts with IIA^{Glc} in *V. vulnificus*

To search for a protein(s) interacting with IIA^{Glc}, *V. vulnificus* crude extract was mixed with His–IIA^{Glc} and subjected to a pull-down assay [22]. Through several independent trials, we could find a protein band migrating with an apparent molecular mass of ~100 kDa that always co-eluted with His–IIA^{Glc} (Fig. 1A). In-gel digestion followed by MALDI-TOF analysis [22] revealed that the protein corresponds to the hypothetical M16 Zn-metallopeptidase family protein vIDE. The vIDE protein has greatest sequence similarity with mammalian insulysins, also known as insulinase or insulin-degrading enzyme, apart from its similarity to its homologs in species closely related to the *Vibrionaceae* family of bacteria (Supplementary Table S1).

To confirm the specific interaction of vIDE with IIA^{Glc}, vIDE was mixed with His–IIA^{Glc} and subjected to a pull-down assay (Fig. 1B). In one set of reactions, glucose was added, along with *E. coli* EI and HPr, to maintain IIA^{Glc} in dephosphorylated form (the reaction mixtures labeled Glc). In another set of reaction mixtures, PEP, EI, and HPr were included to maintain IIA^{Glc} in phosphorylated form (the reaction mixtures labeled PEP). Phosphorylation of IIA^{Glc} was confirmed by its mobility shift in a SDS–PAGE gel [24]. As expected from the Zn-binding nature of the M16 metallopeptidase family proteins, a small fraction of the purified vIDE bound to the metal affinity resin even without His–IIA^{Glc} (Figs. 1B and 2A). However, the amount of the vIDE protein bound to the resin was significantly greater when His–IIA^{Glc} was bound to the affinity resin. Although most regulatory interactions between IIA^{Glc} and its target proteins depend strictly on the phosphorylation state of IIA^{Glc} in *E. coli*

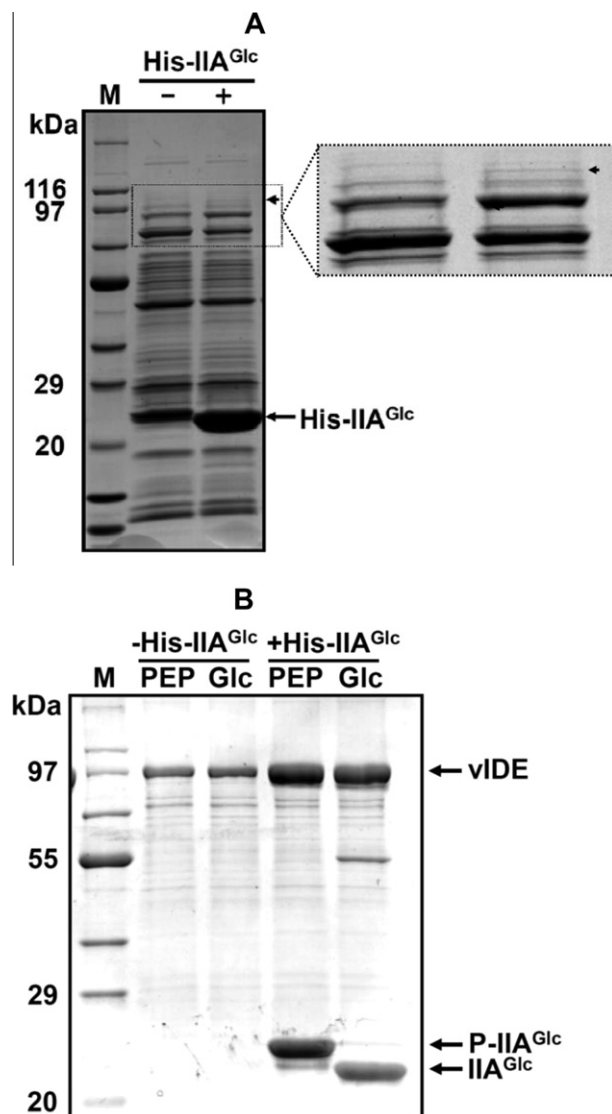


Fig. 1. Specific interaction between IIA^{Glc} and vIDE in *V. vulnificus*. (A) Pull-down assay to search for protein factor(s) interacting with IIA^{Glc} in *V. vulnificus*. Crude extract of *V. vulnificus* MO6-24/O cells was mixed with 2 mg of either purified His-IIA^{Glc} (lane +) or IIA^{Glc} (lane -), and processed as described in Section 2. Aliquots of the eluted protein samples were analyzed by 4–20% gradient SDS-PAGE followed by staining with Coomassie blue. In-gel digestion followed by MALDI-TOF analysis revealed that the protein band specifically bound to His-IIA^{Glc} corresponded to vIDE encoded by *ideV* (marked with an arrow). Lane M, molecular mass markers (Koma Biotech, Inc.). (B) Interaction of IIA^{Glc} with vIDE is independent of its phosphorylation state. vIDE (20 µg) was incubated with EI (3 µg) and HPr (4.5 µg) in the presence or absence of His-IIA^{Glc} (10 µg). Mixtures designated Glc and PEP were supplemented with 1 mM glucose and PEP to ensure complete dephosphorylation and phosphorylation of the added IIA^{Glc}, respectively. The mixtures were then subjected to a pull-down assay using the TALONTM resin. P-IIA^{Glc} stands for the phosphorylated form of His-IIA^{Glc}.

[9,13,14], vIDE interacted with IIA^{Glc} regardless of its phosphorylation state (Fig. 1B).

The concentration dependence of the interaction between IIA^{Glc} and vIDE was studied to measure the affinity of the interaction. While the amount of the vIDE protein bound to the column was independent of the amount of His-EI pre-bound to the affinity resin, it increased as the amount of His-IIA^{Glc} added to the column increased, confirming that vIDE specifically interacts with IIA^{Glc} (Fig. 2A). The K_D was calculated to be about 2.0 µM assuming a 1:1 interaction using Scatchard analysis (Fig. 2B). Noteworthy,

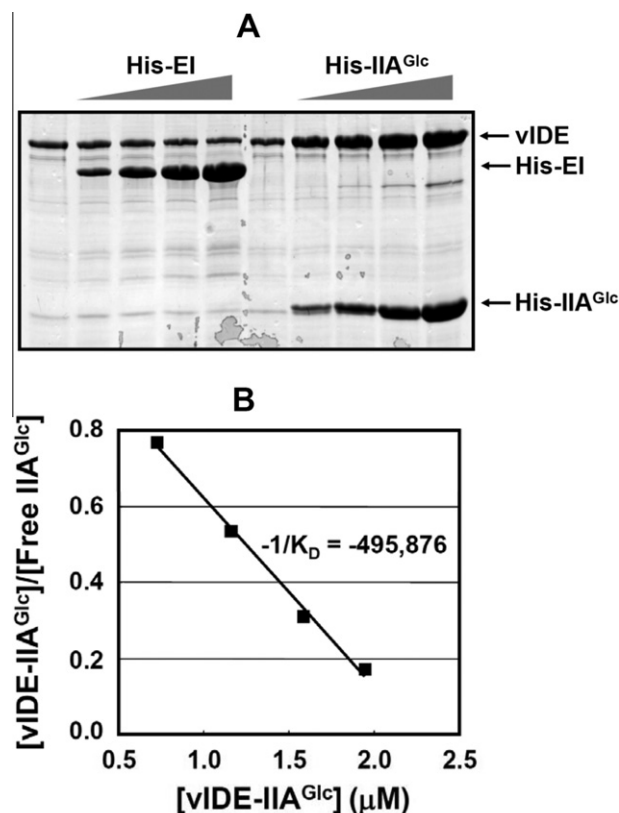


Fig. 2. Kinetics of the interaction between IIA^{Glc} and vIDE. (A) vIDE (20 µg) was incubated with increasing amounts of His-EI or His-IIA^{Glc} (0, 15, 30, 60, and 120 µg) in a total volume of 500 µl and subjected to a pull-down assay. (B) After the SDS-polyacrylamide gel was scanned, the amount of vIDE specifically bound to IIA^{Glc} (bound to the column containing His-IIA^{Glc} minus bound to the control column containing His-EI) was calculated and the concentration of free IIA^{Glc} was obtained by subtracting the amount of bound vIDE from the total IIA^{Glc} for Scatchard analysis.

this binding affinity is in the similar range as reported for other regulatory interactions involving IIA^{Glc} in *E. coli* [12,25,26].

3.2. vIDE activity is dependent on dephosphorylated IIA^{Glc}

A BLAST search revealed that this peptidase is widespread in a variety of organisms including animals, plants, fungi, and protists as well as bacteria (Supplementary Table S1). Amino acid sequence comparison showed that vIDE has an HXXEH active-site motif present in all known members of the Zn-metalloendopeptidase M16 superfamily (Supplementary Fig. S1) [27]. We therefore measured the insulinolytic activity of vIDE and analyzed the effect of IIA^{Glc} on vIDE activity using reverse-phase HPLC [28] (Fig. 3A). Because insulinolysis in other organisms do not require any other protein factors for activity [27], it was surprising that the purified vIDE alone did not show any insulinolytic activity. However, when we added IIA^{Glc} to the reaction mixture together with vIDE, the substrate insulin was completely degraded. IIA^{Glc} alone did not show any insulinolytic activity, implying that the apparent activation of vIDE by IIA^{Glc} does not reflect contamination with proteases during the preparation of IIA^{Glc}. It was previously shown that human insulinolysis is inhibited by the thiol-modifying agent N-ethylmaleimide (NEM) and, to a less extent, by the metal chelator ethylenediaminetetraacetic acid (EDTA) while the insulin-degrading activity of *E. coli* protease III is little affected by NEM but almost completely inhibited by EDTA [29]. Interestingly, the insulin-degrading activity of vIDE was sensitive to NEM as well as EDTA

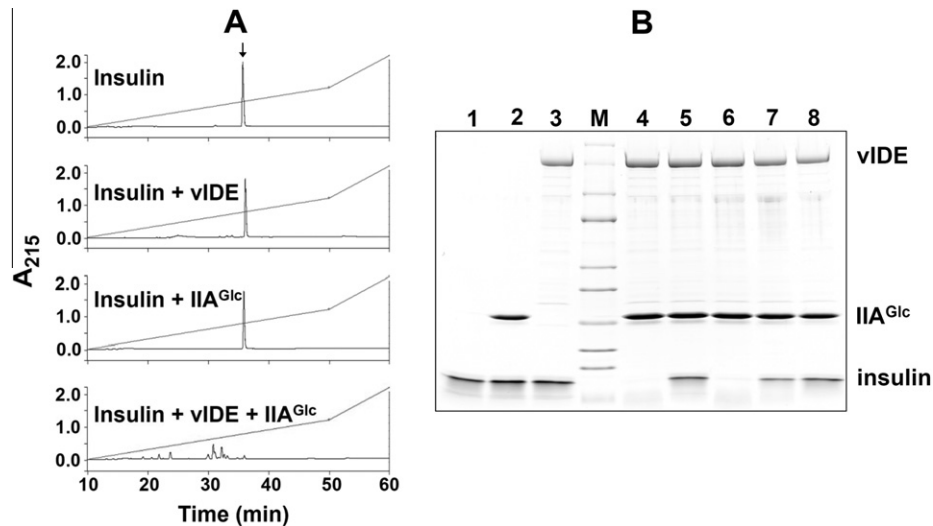


Fig. 3. Insulin-degrading activity of vIDE. (A) Absolute requirement of IIA^{Glc} for the insulinolytic activity of vIDE. Human insulin (Sigma–Aldrich) (designated by an arrow) was incubated with vIDE and/or IIA^{Glc} for 30 min and injected to a C18 Hypersil™ Gold reverse-phase HPLC column after terminating the reaction by adding trifluoroacetic acid. (B) Effect of various chemicals on the insulin-degrading activity of the vIDE-IIA^{Glc} complex. vIDE (1 μ M) was mixed with IIA^{Glc} (2 μ M) in the presence or absence of chemicals (1 mM) and the insulin-degrading reactions were initiated by adding insulin (35 μ M). After 30 min, the reactions were stopped by adding the SDS–PAGE loading buffer and analyzed by 4–20% gradient SDS–PAGE and staining with Coomassie blue. Lane 1, insulin; lane 2, insulin + IIA^{Glc}; lane 3, insulin + vIDE; lane 4, insulin + IIA^{Glc} + vIDE; lane 5, insulin + IIA^{Glc} + vIDE + EDTA; lane 6, insulin + IIA^{Glc} + vIDE + EGTA; lane 7, insulin + IIA^{Glc} + vIDE + NEM; lane 8, insulin + IIA^{Glc} + vIDE + TPEN; lane M, molecular mass markers (Koma Biotech, Inc.).

(Fig. 3B), implying that vIDE has similar biochemical properties with mammalian insulysins. The zinc chelator N,N,N',N'-tetraakis(2-pyridylmethyl)ethylenediamine (TPEN), but not the calcium chelator ethylene glycol tetraacetic acid (EGTA), also inhibited vIDE, indicating that Zn is indispensable for the peptidase activity of vIDE.

Frequently, the phosphorylation state of a regulatory protein modulates the activity of its interacting partners. For example, only the phosphorylated form of *E. coli* IIA^{Glc} can activate adenyl cyclase, while their interaction is independent of the phosphorylation state of IIA^{Glc} [12]. To confirm whether IIA^{Glc} is required for activity of vIDE, and to test whether this activation depends on the phosphorylation state of IIA^{Glc}, the reaction mixture containing vIDE, EI, HPr and IIA^{Glc} was pre-incubated with either PEP or glucose before the reaction was initiated by the addition of insulin. The assay mixture containing phosphorylated IIA^{Glc} did not show any detectable stimulation of insulinolytic activity, while the activity of vIDE was noticeably stimulated by dephosphorylated IIA^{Glc} (Fig. 4A). This result indicates that only dephosphorylated IIA^{Glc} can activate vIDE, although both forms of IIA^{Glc} interact with this protein (Fig. 1B).

The specific activity of vIDE increased hyperbolically with increasing concentration of IIA^{Glc} (Fig. 4B). Maximal specific activity of vIDE against insulin was 7.82 μ moles/min per μ moles of vIDE and half-maximal activation could be obtained in the presence of 1.13 μ M IIA^{Glc}, which is comparable to the K_D of the protein complex (Fig. 2B). Then, we compared the specific activity of vIDE with that of recombinant human insulysin which could degrade insulin without any cofactor. As expected, the insulinolytic activity of human insulysin was little affected by IIA^{Glc}. Intriguingly, vIDE showed about four times higher activity than human insulysin in the presence of IIA^{Glc} (Table 2).

It was previously reported that mammalian insulysins can also degrade other peptides such as amylin, β -amyloid protein and β -endorphin [30,31]. We found that vIDE can also degrade other peptides such as β -amyloid protein and β -endorphin in addition to insulin in the presence of IIA^{Glc} (Fig. 5). These data indicate that vIDE needs to make a complex with IIA^{Glc} to catalyze its peptidase

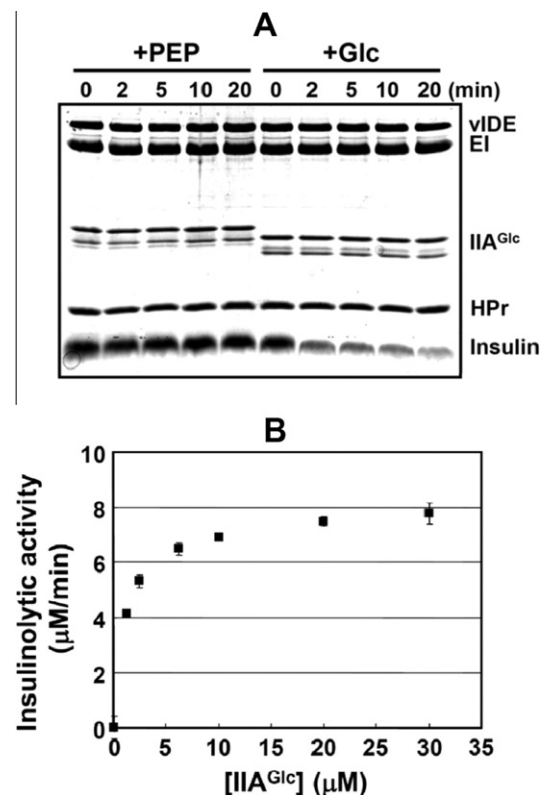


Fig. 4. Phosphorylation state-dependent activation of vIDE by IIA^{Glc}. (A) Phosphorylation of IIA^{Glc} abolishes its stimulatory activity. Insulin (40 μ M) was incubated with EI (2 μ M), HPr (5 μ M), and IIA^{Glc} (2 μ M) in the presence of 1 mM PEP or glucose. At the indicated time points after addition of vIDE (1 μ M), aliquots were mixed with 2 \times SDS–PAGE loading buffer and resolved on a 20% SDS–PAGE gel. (B) Specific activity of vIDE in the presence of various concentrations of IIA^{Glc}. The degradation of insulin by vIDE (1 μ M) was measured in the presence of IIA^{Glc} at the indicated concentrations. Reactions were incubated for 10 min and stopped by adding 1/10 volume of 5.5% trifluoroacetic acid (TFA) and the amounts of remaining insulin were quantified by HPLC.

Table 2

Comparison of insulinolytic activities between vIDE and human insulin. vIDE and recombinant human insulin (1 μM each) in the presence or absence of IIA^{Glc} (20 μM) was incubated with human insulin (100 μM, Sigma–Aldrich) in 100 mM Tris–HCl (pH 7.5) at 30 °C (n = 3). Reactions were terminated by adding 1/10 volume of 5.5% trifluoroacetic acid and the degradation of insulin was measured through HPLC.

	Specific activity (μmoles of insulin/min/μmoles of enzyme)	
	vIDE	Human insulin
– IIA ^{Glc}	No activity	2.22 ± 0.21
+ IIA ^{Glc}	7.82 ± 0.34	2.18 ± 0.13

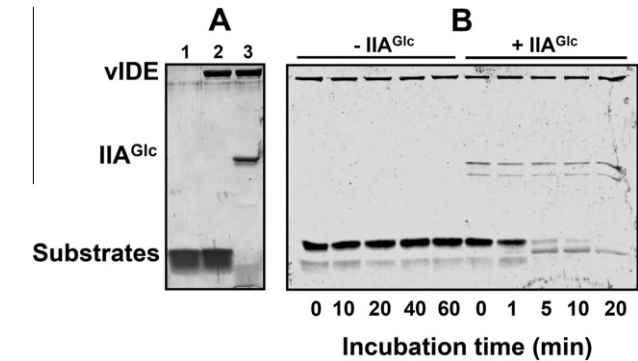


Fig. 5. Hydrolysis of β-endorphin and β-amyloid by vIDE. (A) vIDE was mixed with β-endorphin and its hydrolytic activity was measured after incubation for 30 min: Lane 1, β-endorphin alone; lane 2, β-endorphin + vIDE; lane 3, β-endorphin + vIDE + IIA^{Glc}. (B) vIDE was mixed with β-amyloid in the presence (+) or absence (–) of IIA^{Glc} and incubated for the indicated time periods to measure its hydrolytic activity. Samples were run on a 20% SDS–PAGE gel and stained with Coomassie blue.

activity regardless of substrates and support that vIDE in a complex with IIA^{Glc} has similar biochemical characteristics with mammalian insulysins.

3.3. vIDE affects survival and virulence of *V. vulnificus* in mice

To address the role of vIDE in *V. vulnificus*, we compared growth of an *ideV* deletion mutant with that of its parental strain in a set of 20 96-well microplates containing different nutrients and inhibitors by employing Phenotype MicroarrayTM (Biolog Co.). Out of nearly 2000 cellular phenotypes examined, only 3 parameters were observed as meaningfully different in the *ideV* mutant in two independent experiments (see [Supplementary Fig. S2](#)). When we compared growth of the two strains in flasks, however, none of these phenotypes could be verified as significantly different in the mutant. Furthermore, we could find no additional phenotypes resulting from the *ideV* mutation with respect to colony morphology and growth at different temperatures in different media regardless of whether glucose was present.

Then, we assumed that vIDE might be necessary for infection of this pathogenic bacteria to animal hosts. Therefore, we sought to determine whether vIDE plays a role in virulence in a mouse model. Seven-week-old ICR mice were infected with ~1.5 × 10⁴ cells of the wild-type or *ΔideV* *V. vulnificus* strains. This number of cells was chosen because it is about 10-fold higher than the wild-type LD₅₀ determined in pre-test [32]. All the mice injected with wild-type *V. vulnificus* cells died within 15 h, whereas those injected with *ΔideV* cells survived well (Fig. 6). From this result, we assumed that deletion of the *ideV* gene might influence its survival efficiency in the infected mice. Therefore, survival of this pathogen in mice was measured. Two hours after co-infection of equal num-

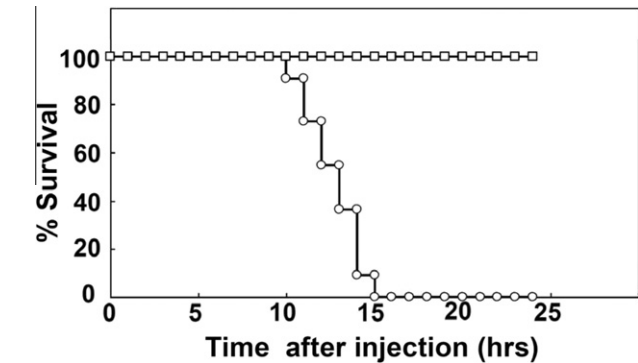


Fig. 6. Effect of vIDE on virulence of *V. vulnificus*. The iron dextran treated seven-week-old female ICR mice were intraperitoneally injected with ~1.5 × 10⁴ cells of wild-type *V. vulnificus* (circles) or *ΔideV* mutant (squares) to test their virulence (P < 0.0001; n = 11 each).

Table 3

Effect of vIDE on survival of *V. vulnificus* in host. Equal numbers of wild-type and *ΔideV* cells (3 × 10⁵ per each strain) were intraperitoneally injected into female ICR mice (n = 3). The homogenates of livers and spleens extracted 2 h after injection were serially diluted in PBS and colony-forming units (CFU) were determined on the TCBS agar plates. The fraction of the mutant cells was estimated by enumerating kanamycin-resistant colonies.

	MO6-24/O	<i>ΔideV</i>
Spleen	35,920 ± 1245	1350 ± 495
Liver	3300 ± 141	Not detected

bers of wild-type and the *ΔideV* mutant, the viable numbers of each bacterial strain were determined in livers and spleens of the infected mice. The competitive index of *ΔideV* mutant compared to wild-type was less than 0.04 in both spleen and liver (Table 3). These results demonstrate that vIDE is important for survival of this pathogen during host infection.

4. Discussion

Bacteria have the capacity to efficiently adapt to environmental changes by re-directing metabolic pathways. The carbohydrate PTS plays an important role in re-directing carbohydrate metabolisms by monitoring a certain carbon source from the environment [6]. In addition to its role in sugar transport, the carbohydrate PTS has many secondary functions. It switches on and off the utilization of a large number of carbon sources in response to the availability of glucose, primarily via controlling the state of phosphorylation of IIA^{Glc}. In this study, we show for the first time that IIA^{Glc} regulates the activity of a protease homologous to mammalian insulysin in *V. vulnificus* by direct interaction. While the specific interaction of IIA^{Glc} with the insulysin homolog is independent of the phosphorylation state of IIA^{Glc}, the peptidase activity of the insulysin homolog is strictly dependent on the unphosphorylated form of IIA^{Glc} (Figs. 1B and 4A). In addition to the insulin, it is able to degrade other mammalian insulysin substrates such as β-endorphin and β-amyloid (Fig. 5). Taken together, our results suggest that the vIDE–IIA^{Glc} complex exhibits similar biochemical properties to the mammalian insulysins and could act by sensing glucose in the environment.

Estuarine seawaters, which are natural habitats for *V. vulnificus*, are usually oligotrophic and thus lack glucose. Upon infection, the bacterial cells are exposed to the relatively eutrophic milieu. One possible way for a bacterial pathogen to recognize the host

environment might be accomplished by sensing chemicals such as glucose. A variety of regulatory functions exerted by the bacterial PTS depend on the phosphorylation states of the involved components, and the cellular fraction of the unphosphorylated forms of the PTS components increases in the presence of a sugar substrate of the PTS [6]. Therefore, it could be predicted that the PTS sugar glucose in the host blood serum would influence the physiological state and thus survival of the pathogens exhibiting tropism for blood vessels and spreading intravascularly such as *V. vulnificus* [33]. In this study, we supported this prediction by showing attenuated virulence and host colonization of a mutant deficient in vIDE whose activity is strictly dependent on the unphosphorylated form of IIA^{Glc} of the PTS (Fig. 6 and Table 3). Recently, relationship between carbohydrate utilization and virulence has been studied in several human pathogenic bacteria such as *Listeria monocytogenes*, *Bacillus anthracis* and group A *Streptococcus* [34–36]. In these species, the change of environmental carbohydrates acts as a signal to control their virulence gene expression through the regulation of a virulence transcription factor. In case of *V. vulnificus*, however, we hypothesize that the PTS senses glucose in host to activate vIDE which plays a role in survival during infection.

Although insulin is a central regulator of glucose homeostasis in mammals and we provide several lines of evidences showing that the vIDE-IIA^{Glc} complex has similar biochemical properties with mammalian insulins (Figs. 3B, 5, and S1 and Table S1), it still remains to be clarified how vIDE regulates survival of *V. vulnificus* cells in mice. vIDE and IIA^{Glc} were found exclusively in the cytoplasm and, compared with the $\Delta ideV$ strain, wild-type *V. vulnificus* showed no significant difference in the insulin-degrading activity unless cells were disrupted (data not shown). Therefore, more experiments are needed to identify the real substrates of vIDE that might regulate adaptation of this pathogen to the host environment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.10.035.

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