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Functional and Phylogenetic Characterization of Vaginolysin, the Human-Specific Cytolysin from *Gardnerella vaginalis*▽†

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Pore-forming toxins are essential to the virulence of a wide variety of pathogenic bacteria. *Gardnerella vaginalis* is a bacterial species associated with bacterial vaginosis (BV) and its significant adverse sequelae, including preterm birth and acquisition of human immunodeficiency virus. *G. vaginalis* makes a protein toxin that generates host immune responses and has been hypothesized to be involved in the pathogenesis of BV. We demonstrate that *G. vaginalis* produces a toxin (vaginolysin [VLY]) that is a member of the cholesterol-dependent cytolysin (CDC) family, most closely related to intermedilysin from *Streptococcus intermedius*. Consistent with this predicted relationship, VLY lyses target cells in a species-specific manner, dependent upon the complement regulatory molecule CD59. In addition to causing erythrocyte lysis, VLY activates the conserved epithelial p38 mitogen-activated protein kinase pathway and induces interleukin-8 production by human epithelial cells. Transfection of human CD59 into nonsusceptible cells renders them sensitive to VLY-mediated lysis. In addition, a single amino acid substitution in the VLY undecapeptide [VLY(P480W)] generates a toxoid that does not form pores, and introduction of the analogous proline residue into another CDC, pneumolysin, significantly decreases its cytolytic activity. Further investigation of the mechanism of action of VLY may improve understanding of the functions of the CDC family as well as diagnosis and therapy for BV.

Bacterial vaginosis (BV) is a common and incompletely understood condition associated with adverse outcomes including preterm birth and acquisition of sexually transmitted diseases (10). Alterations of both local host immunity and the genital tract microflora appear to contribute to the pathogenesis of BV (39), and BV can be difficult to eradicate even in the setting of targeted antimicrobial therapy (4). In addition, randomized trials of antibiotics for the prevention of BV-associated preterm birth have not shown consistently beneficial effects, suggesting that host inflammatory responses set in motion early in the course of disease may contribute significantly to the consequences of infection (27).

In the 1950s, Leopold (25) and then Gardner and Dukes (14) observed abundant small, pleomorphic gram-variable rods in the genital tract of women with BV. This organism, first called *Haemophilus vaginalis* (13) and repeatedly renamed as more information about its characteristics became available (reviewed in reference 5), is now classified as *Gardnerella vaginalis*, the sole member of the genus *Gardnerella* (16, 30). Phylogenetic analysis based on 16S rRNA places *Gardnerella* in the gram-positive family *Bifidobacteriales*. An abundance of *G. vaginalis* and a paucity of *Lactobacillus* species are characteristic of a BV-associated microflora, but the relative contribution of *G. vaginalis* to the pathogenesis of BV is not clear. *G. vaginalis* is present in essentially all cases of BV but can also be detected in a minority of asymptomatic women (1). Likewise, using molecular techniques, several groups have demonstrated that the vaginal microflora is exceedingly complex and that in the setting of BV the vaginal mucosa is host to many non-*Gardnerella* organisms (12, 18, 20). Mechanistic studies of BV and its adverse consequences have been limited by the absence of definitive diagnostic testing and a suitable animal model (22, 23, 26).

G. vaginalis produces a protein toxin that acts as a hemolysin (8, 35). Immunoglobulin A-mediated immune responses to the hemolysin occur during BV and are useful as a marker of disease (8, 35). Complete characterization of the hemolysin has been limited by the absence of genetic information and an inability to produce recombinant toxin. Here we demonstrate that the *G. vaginalis* hemolysin is a member of the cholesterol-dependent cytolysin (CDC) family of toxins and suggest the name vaginolysin (VLY) for consistency with CDC nomenclature. Similar to intermedilysin (ILY) (15, 29), VLY is selective for human cells, and host specificity occurs through recognition of the complement regulatory molecule CD59. A proline residue in the undecapeptide of domain 4 of VLY is critical for VLY-mediated pore formation and cytotoxicity, and mutation of this residue generates a VLY toxoid that may be useful in vaccine development.

MATERIALS AND METHODS

Bacterial strains and cell lines. *Gardnerella vaginalis* strains 14018, 14019, and 49145 were obtained from the ATCC and grown in brain heart infusion supplemented with 5% fetal bovine serum, 0.3% Tween 80, and 0.1% soluble starch. *Escherichia coli* strains TOP10 and BL21AI (Invitrogen) were grown in LB, with kanamycin (30 μg/ml) selection as appropriate. HeLa cells were grown at 37°C and 5% CO_2 in minimal essential medium supplemented with 10% fetal bovine serum and 10 μg/ml ciprofloxacin. CHO-K1 cells (CCL-61) were grown at 37°C

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and 5% CO_2 in F-12 Kaighn's modification (Invitrogen) with 10% fetal bovine serum and 10 μg/ml ciprofloxacin.

Cloning, sequencing, and analysis of the VLY gene. The *G. vaginalis* genomic region containing VLY was amplified from *G. vaginalis* 14018 by PCR using *Pfx* proofreading polymerase (Invitrogen) and primers V1 (ATGCAGCGAAGCA TGCCATGC) and V2 (TCAGTCGTTCTTTACAGTTTC). This PCR product was cloned into vector pCR2.1/TOPO (Invitrogen) and transformed into *E. coli* TOP10 according to the manufacturer's instructions. The insert was bidirectionally sequenced using vector-specific primers. The predicted VLY open reading frame was amplified by PCR using the cloned genomic region as template, *Pfx* polymerase, and primers V3 (GCCGCCGCCCATATGAAGAGTACAAAG) and V6 (GCCGGATCCTCAGTCGTTCTTTACAGT), adding unique restriction sites indicated by underlining. The resulting product was cut with restriction enzymes NdeI and BamHI, cloned into the vector pET28a (Novagen) to generate a construct with an N-terminal hexahistidine transcriptional fusion, and confirmed by sequencing. Site-directed mutagenesis to construct pET28a/VLY(P480W) was performed with the QuikChange II XL kit (Stratagene) according to the manufacturer's instructions. Mutagenic primers used were P480Wsense (TGGTTGAAAAGACTGGTTTGGTTTGGGAATGGTGGCG CACAGTATAT) and P480Wanti (ATATACTGTGCGCCACCATTCCCAAA CCAAACCAGTCTTTTCAACCA).

Protein sequence prediction, alignment, and phylogenetic analyses were performed using MacVector software (version 9.5; MacVector Inc.). Protein sequences for other CDC family members were obtained from the Comprehensive Microbial Resource (J. Craig Venter Institute, http://cmr.tigr.org) or from the GenBank/Entrez Protein database (National Center for Biotechnology Information).

Expression and purification of recombinant toxins. *E. coli* BL21AI carrying the pET28a/VLY or pET28a/VLY(P480W) plasmid was grown in 1-liter cultures at 37°C on a rotary shaker for 3 h, and protein expression was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and 0.02% L-arabinose (Sigma). After 6 h, bacteria were pelleted and lysed with BugBuster solution (Novagen) in the presence of protease inhibitor cocktail, lysozyme (100 μg/ml), and benzonase nuclease, all from Sigma. Lysates were cleared by centrifugation, and tagged recombinant toxin was purified using nickel-nitrilotriacetic acid agarose (Qiagen) according to the manufacturer's instructions. Purified toxin was extensively dialyzed against lipopolysaccharide-free phosphate-buffered saline (PBS) (Gibco) to remove imidazole and concentrated (Amicon Ultra; 10-kDa-molecular-mass cutoff). Protein concentrations were determined using a modified Bradford assay (Bio-Rad).

The coding sequence of the pneumolysin (PLY) gene was amplified by PCR using primers NdeI-Ply-up (GGAATTCCATATGGCAAATAAAGCAG) and Ply-down-XhoI (CCGCTCGAGGTCATTTTCTACCTTATC) using genomic DNA of *Streptococcus pneumoniae* strain TIGR4 as a template. These primers added unique restriction sites as indicated by underlining and led to amplification of the entire PLY sequence, omitting the stop codon to allow addition of a C-terminal hexahistidine tag. The product was confirmed by sequencing, digested with NdeI and XhoI (New England Biolabs), and cloned into pET29a (Novagen) cut with NdeI and XhoI. The plasmid was transformed into *E. coli* BL21AI, and induction and purification were performed as described for VLY. Site-directed mutagenesis used primers W435Psense (ACCGGGCTTGCCTGGGAACCGT GGCGTACG) and W435Panti (CGTACGCCACGGTTCCCAGGCAAGCCC GGT).

Anti-PLY Western blot assay. *G. vaginalis* 14018 was grown on chocolate agar, and fresh colonies were scraped from the plate and resuspended in lysis buffer (BugBuster; EMD Chemicals, Gibbstown, NJ) with benzonase nuclease. The lysate was boiled for 5 min, and 30 μl of lysate was separated on a 4 to 12% polyacrylamide gel (Invitrogen). Purified VLY (500 ng total) was run as a positive control. The proteins were transferred to polyvinylidene difluoride membranes, blocked with 5% milk, and probed with murine monoclonal anti-PLY (clone 9.1/2/3/6; Novocastra, Newcastle Upon Tyne, United Kingdom; 1:100 dilution). Detection was with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (Santa Cruz Biotechnology, Santa Cruz, CA) and enhanced chemiluminescence.

Erythrocyte lysis assay. The use of human erythrocytes was approved by the Columbia University Institutional Review Board. Human blood was obtained by venipuncture, and erythrocytes were immediately isolated by centrifugation and repeated washing in sterile PBS. Blood from other species tested was obtained commercially (Fisher Scientific), and erythrocytes were washed in sterile PBS prior to use. A 1% solution of packed erythrocytes in sterile PBS was combined with an equal volume of toxin diluted in PBS. The total volume for the assay was 200 μl per well of a 96-well polystyrene V-bottomed plate. The negative control for lysis consisted of PBS without toxin added to erythrocytes, and the positive control for 100% lysis was 0.05% Triton X-100. Incubation was for 30 min at 37°C and 5% CO_2. At the conclusion of the assay, the plates were spun at 2,000 rpm to pellet erythrocytes, and supernatants were removed for measurement of optical density at 415 nm. Where noted, toxins were preincubated with cholesterol (stock solution, 100 mg/ml in chloroform; working concentration, 1 to 10 μg/ml) or control (chloroform alone at the corresponding dilution) for 10 min at room temperature prior to use in the assay. Antibody inhibition experiments were performed using anti-CD55 (clone IA10; BD Pharmingen), anti-CD59 (clone YTH53.1; GeneTex), or irrelevant antibody control. Preincubation of erythrocytes with antibody (9-ng/ml final concentration) was for 1 h at 4°C with constant rotation, followed by two PBS washes to remove unbound antibody prior to use in the assay.

CHO cell transfection and lactate dehydrogenase release assay. The coding sequence for human CD59 was amplified from cDNA from A549 (CCL-185) respiratory epithelial cells using primers CD59-1 (GCCGCCCTCGAGCCACC AATGGGAATCCAAGGAG) and CD59-2 (GCCGCCGAATTCTTAGGGAT GAAGGCTCCAGGC) and cloned into the XhoI and EcoRI sites of pIRES2-EGFP (Clontech). Sequence was confirmed using vector-specific primers. CHO-K1 cells were transfected with purified plasmid DNA (either pIRES2-EGFP/CD59 or the corresponding empty vector control) by using a Nucleofector (Amaxa) according to the manufacturer's instructions. Transfected cells were plated into six-well dishes and used 48 h after transfection. Greater than 90% transfection efficiency was assessed by fluorescence microscopy (data not shown). Cells were weaned from serum overnight and stimulated with VLY or PLY diluted in serum-free F-12 medium for 30 min at 37°C and 5% CO_2. Cell viability was confirmed at the end of the experiment by visual inspection of the monolayer and trypan blue exclusion and exceeded 90%. The positive control for complete lysis was 1% Triton X-100 in serum-free F-12. The concentration of lactate dehydrogenase in supernatants was assessed with a commercial kit (Roche) according to the manufacturer's instructions.

Epithelial p38 MAPK phosphorylation. Western blot analysis of epithelial p38 mitogen-activated protein kinase (MAPK) phosphorylation was performed as previously described (32).

Real-time PCR. HeLa cells were weaned from serum overnight and treated for 2 h with medium alone, VLY (10 μg/ml), or VLY(P480W) (10 μg/ml). Cells were lysed in RLT+ buffer (Qiagen) and RNA purified using a commercially available kit (RNeasy Plus; Qiagen). Reverse transcription of 1.5 μg of RNA per sample to generate cDNA was performed with the high-capacity cDNA kit (Applied Biosystems). Real-time PCR (Applied Biosystems StepOne) with Sybr green detection (PowerSYBR; Applied Biosystems) was performed using primers for interleukin-8 (IL-8) (TACTCCAAACCTTTCCAACCC and AACTTCTCCAC AACCCTCTG) and glyceraldehyde-3-phosphate dehydrogenase (GGGCGCCT GGTCACCAGGGCTG and GGGGCCATCCACAGTCTTCTG). Relative quantitation used the cycle threshold method with normalization to glyceraldehyde-3-phosphate dehydrogenase.

Statistical analysis. Statistical comparisons were performed using two-tailed unpaired *t* tests or one-way analysis of variance (ANOVA) with Tukey posttest as appropriate (Prism; GraphPad Software).

Nucleotide sequence accession numbers. The sequence data for VLY from *G. vaginalis* strains 14018, 14019, and 49145 are available in GenBank under the accession numbers EU522486 to EU522488.

RESULTS

The *G. vaginalis* genome contains an orthologue of known CDCs. The CDC family is made up of more than 15 protein toxins produced by several distinct gram-positive genera (reviewed in reference 38). The basic local alignment search tool (BLAST) was used to compare raw genomic DNA sequence data from the *Gardnerella vaginalis* 14018 genome project (available at http://med.stanford.edu/sgtc/research/gardnerella_vaginalis.html) with a database of known microbial genes (Comprehensive Microbial Resource, J. Craig Venter Institute). A 1,551-bp open reading frame with 54% DNA sequence identity to PLY, the *S. pneumoniae* CDC, was identified. This genomic region was amplified from *G. vaginalis* 14018 (ATCC) by PCR, cloned, and sequenced.

The predicted amino acid sequence of VLY exhibits sequence similarity and identity consistent with reported rela-

TABLE 1. Comparison of the predicted primary amino acid sequence of VLY with other CDCs

CDC[a]	% Identity	% Similarity
ILY	55.1	73.9
PLY	47.9	65.9
MLY	47.7	65.7
SUI	47.5	63.5
PYO	38.9	55.0
TET	37.8	57.7
IVN	37.8	55.8
LLO	36.6	56.3
PFO	36.6	55.9
SLG	36.4	55.7
THU	35.9	54.5
ALO	35.9	54.3
CER	35.5	54.5
ALV	35.3	56.3
SPH	33.8	54.8
SLO	31.9	51.2

[a] Abbreviations: MLY, mitilysin; SUI, suilysin; PYO, pyolysin; TET, tetanolysin O; IVN, ivanolysin; LLO, listeriolysin O; PFO, perfringolysin O; SLG, seeligeriolysin; THU, thuringensolysin; ALO, anthrolysin O; CER, cereolysin; ALV, alveolysin; SPH, sphaericolysin; SLO, streptolysin O.

tionships among members of the CDC family (Table 1). The predicted sequence of VLY from *G. vaginalis* 14019 is identical to the sequence from strain 14018. A third *G. vaginalis* strain (ATCC 49145) is identical with the exception of a single amino acid substitution (R494H). A phylogram of representative full-length CDC sequences (Fig. 1A) obtained from publicly available databases (Table 2) was constructed using the neighbor joining algorithm. By this analysis, VLY appears to be most closely related to ILY and to fall within a group consisting of most of the CDCs from the genus *Streptococcus*, including PLY, mitilysin, and suilysin. Pyolysin, from *Arcanobacterium pyogenes* (2), is the least similar member of this group. VLY is more distantly related to CDCs from the *Bacillus*, *Listeria*, and *Clostridium* genera, as well as streptolysin O from *Streptococcus pyogenes*, which is divergent from the other streptococcal CDCs. Bootstrap analysis indicates a high degree of confidence for the placement of VLY in the streptococcal group (not shown).

The undecapeptide, an 11-amino-acid sequence in domain 4 of the CDCs, is a well-conserved region of particular importance for host cell interaction and pore formation (38). The VLY undecapeptide is divergent from the CDC consensus sequence at 3 of 11 sites (Fig. 1B), one of which is an alanine-to-valine alteration. More strikingly, there is a proline substitution at VLY position 480, the site of one of the conserved tryptophan residues important for pore formation in other CDC family members (24). The loss of the conserved cysteine residue at the second position of the undecapeptide is seen in two other CDCs, ILY and pyolysin, and is consistent with the prior report of insensitivity of the *G. vaginalis* toxin to reducing agents (35). Western blot analysis of lysed *G. vaginalis* bacteria demonstrates a ~57-kDa band that cross-reacts with a monoclonal antibody directed against the *S. pneumoniae* CDC, PLY. Purified, recombinant VLY migrates at a similar molecular weight and is also detected by anti-PLY antibody (Fig. 1C).

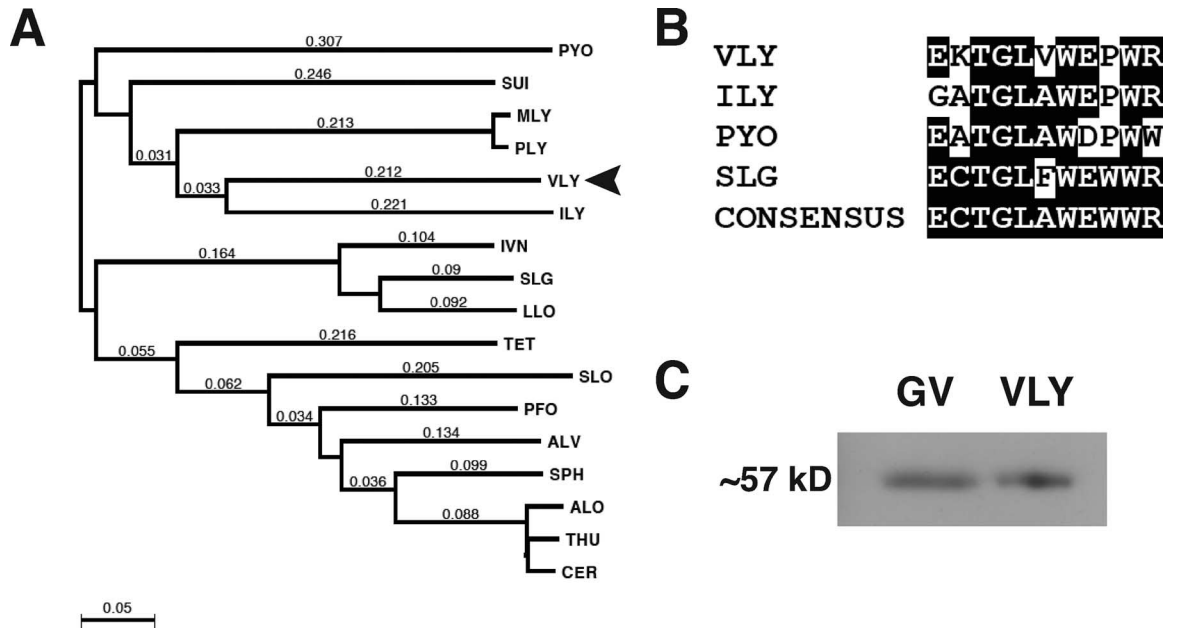

FIG. 1. Phylogenetic relationship between VLY and other members of the CDC family. (A) Phylogram of full-length CDC protein sequences predicted by the neighbor joining algorithm. Numbers represent calculated relative phylogenetic distances. Abbreviations for CDC proteins: LLO, listeriolysin O; IVN, ivanolysin; SLG, seeligeriolysin; SPH, sphaericolysin; ALO, anthrolysin O; CER, cereolysin; PFO, perfringolysin O; ALV, alveolysin; TET, tetanolysin O; PYO, pyolysin; MLY, mitilysin; SLO, streptolysin O; SUI, suilysin; THU, thuringensolysin. (B) Multiple alignment of undecapeptide regions from known CDCs. The predicted amino acid sequence of VLY contains a variant undecapeptide region most similar to the undecapeptide from ILY. The sequence labeled "consensus" corresponds to the undecapeptide from MLY, PLY, SUI, IVN, ALV, SPH, THU, SLO, ALO, LLO, PFO, CER, and TET. (C) Western blot of lysed *G. vaginalis* bacteria (GV) and purified, recombinant VLY, probed with anti-PLY monoclonal antibody.

TABLE 2. Sources of sequence data for phylogenetic analyses

Name	Abbreviation	Bacterial strain	Sequence source[a]	Accession no.
Pneumolysin	PLY	*Streptococcus pneumoniae* TIGR4	CMR	SP_1923
Listeriolysin O	LLO	*Listeria monocytogenes* EGD-e	CMR	LMO0202
Intermedilysin	ILY	*Streptococcus intermedius* UNS38	GEN	BAE16324
Ivanolysin	IVN	*Listeria ivanovii* NRRL 33017	GEN	AAR97343
Seeligeriolysin	SLG	*Listeria seeligeri* NRRL 33019	GEN	AAR97361
Sphaericolysin	SPH	*Lysinibacillus sphaericus* A3-2	GEN	AB273179
Anthrolysin O	ALO	*Bacillus anthracis* Ames	CMR	BA_3355
Cereolysin O	CER	*Bacillus cereus* ATCC 14579	CMR	BC_5101
Perfringolysin O	PFO	*Clostridium perfringens* 13	CMR	CPE0163
Alveolysin	ALV	*Paenibacillus alvei*	GEN	P23564
Tetanolysin O	TET	*Clostridium tetani* E88	CMR	CTC_01888
Pyolysin	PYO	*Arcanobacterium pyogenes* BBR1	GEN	AAC45754
Mitilysin	MLY	*Streptococcus mitis* 990123	GEN	ABK58690
Streptolysin O	SLO	*Streptococcus pyogenes* SSI-1	CMR	SPS0132
Suilysin	SUI	*Streptococcus suis* 3	GEN	CAC94852
Thuringensolysin	THU	*Bacillus thuringensis* Konkukian	CMR	BT9727_3096
Vaginolysin	VLY	*Gardnerella vaginalias* 14018	This study	

[a] CMR, Comprehensive Microbial Resource (http://www.tigr.org); GEN, GenBank (http://www.ncbi.nlm.nih.gov/GenBank).

Species-specific, cholesterol-dependent hemolytic activity of VLY. Recombinant VLY produced in *E. coli* was used for studies of toxin interaction with target cells. Purified VLY lysed primary human erythrocytes in a dose-dependent fashion (Fig. 2A). In contrast, sheep (Fig. 2A and B), mouse (Fig. 2B), and horse (data not shown) erythrocytes were resistant to lysis even at substantially higher VLY concentrations. Erythrocytes from all of these species were lysed by PLY, a non-species-restricted member of the CDC family (Fig. 2B). Preincubation of VLY with cholesterol inhibited lysis of human erythrocytes in a dose-dependent manner, consistent with its classification within the CDC family (Fig. 2C).

Host specificity of VLY depends on the complement regulatory molecule CD59. The human-specific action of VLY is reminiscent of findings with ILY, a member of the CDC family that specifically binds human CD59 as a requirement for lytic activity (15). Monoclonal antibody-mediated blockade of CD59 on the surface of primary human erythrocytes abrogated

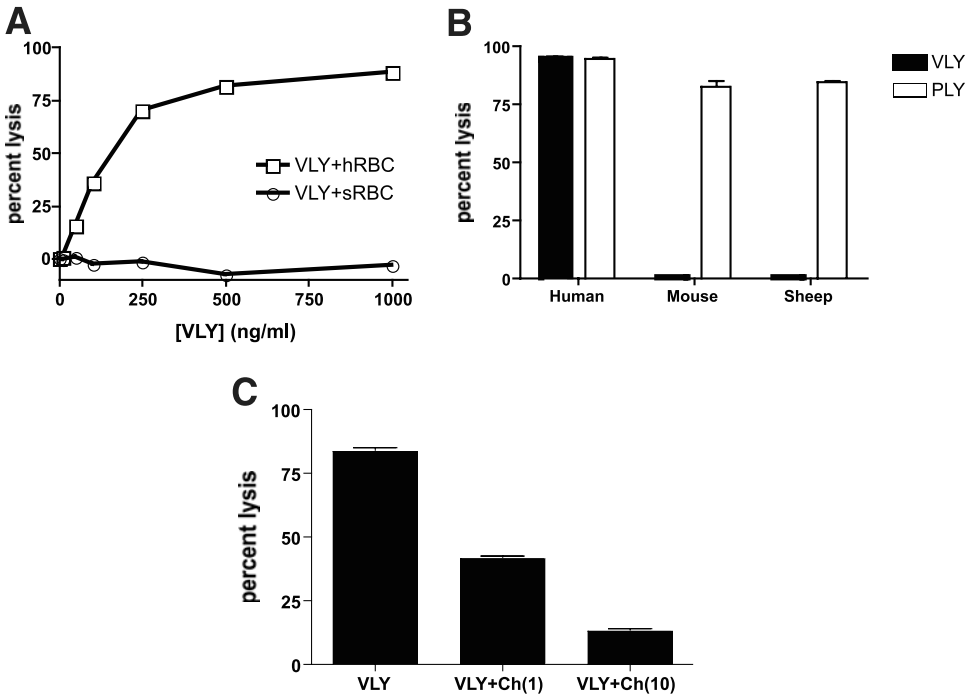

FIG. 2. Human-specific, cholesterol-dependent hemolytic activity of VLY. (A) Washed human (hRBC) or sheep (sRBC) erythrocytes (1% solution in PBS) were exposed to the indicated concentrations of purified recombinant VLY for 30 min, followed by pelleting of cells. Hemoglobin release was measured as optical density at 415 nm of the supernatant and normalized to 100% lysis for each species tested ($P < 0.01$, ANOVA). (B) Erythrocytes from various species were exposed to VLY or the non-species-specific toxin PLY (both toxins at 5 μg/ml), and lysis was measured. (C) Addition of cholesterol (Ch) at 1 μg/ml or 10 μg/ml inhibits human erythrocyte lysis by VLY (5 μg/ml) ($P < 0.001$, ANOVA).

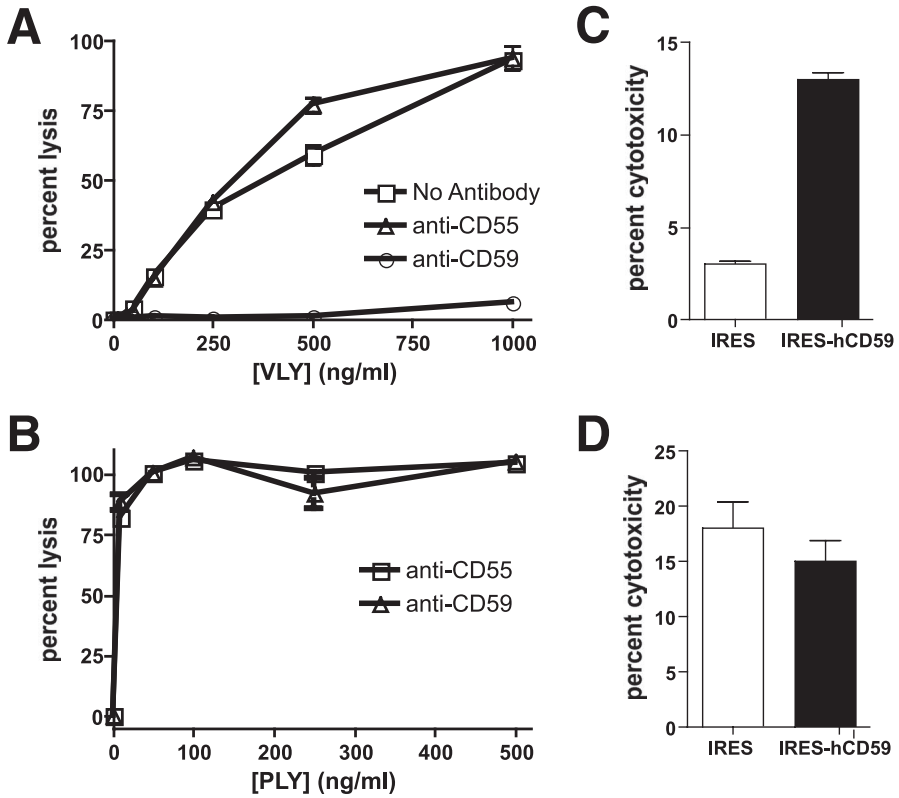

FIG. 3. Host specificity of VLY depends on the complement regulatory molecule CD59. (A) VLY-induced lysis of human erythrocytes was inhibited by monoclonal antibody to human CD59 ($P < 0.0001$) but not antibody to another glycosylphosphatidylinositol-anchored cell surface antigen (CD55) or mock treatment (PBS). (B) Antibody to CD59 does not inhibit PLY-mediated lysis of human erythrocytes. (C) Lactate dehydrogenase release from Chinese hamster ovary (CHO) cells transfected with empty vector (IRES) or human CD59 (IRES-hCD59) and exposed to VLY (10 μg/ml) for 30 min. Transfection of human CD59 increases VLY-mediated lysis ($P < 0.0001$). (D) Transfection of human CD59 into CHO cells does not affect PLY (1 μg/ml)-mediated lysis ($P > 0.05$).

VLY-induced lysis, whereas antibody against another erythrocyte surface marker, CD55, was ineffective (Fig. 3A). In contrast, the activity of PLY, a CDC that does not exhibit host specificity, was not inhibited by either antibody (Fig. 3B). Transfection of human CD59 into Chinese hamster ovary (CHO) cells significantly increased lactate dehydrogenase release in the setting of treatment with VLY (Fig. 3C) but not PLY (Fig. 3D), indicating that CD59 is sufficient to confer susceptibility on at least a subset of VLY-resistant cells.

VLY activates epithelial cell proinflammatory signaling. Although hemolysis is a useful model for toxin-induced pore formation, erythrocytes are unlikely to be a target cell for *G. vaginalis* under normal physiologic conditions, as *Gardnerella* bacteremia is exceedingly rare (11, 33). Activation of p38 MAPK is a conserved element in epithelial detection of bacterial pore-forming toxins (32) and appears to be essential in defense of host cells from toxin attack (19). Exposure of the human cervical epithelial cell line HeLa to VLY led to phosphorylation of p38 MAPK within 30 min (Fig. 4A), consistent with epithelial responses to other pore-forming toxins (32). In addition, treatment of HeLa cells with VLY but not VLY(P480W) led to upregulation of mRNA for IL-8, the major neutrophil chemokine, as assessed by real-time reverse transcription-PCR (Fig. 4B).

The proline residue in the variant undecapeptide of VLY is required for cytolytic and cell stimulatory activity. Mutation of conserved tryptophan residues in the CDC undecapeptide can have drastic effects on the efficiency of pore formation (24). We hypothesized that the tryptophan-to-proline mutation at position 480 in VLY would have effects on host range and cytolytic and cell stimulatory activity. Site-directed mutagenesis allowed expression and purification of the single-amino-acid mutant protein VLY(P480W). This proline-to-tryptophan mutation abolished p38 activation and IL-8 transcription in HeLa cells (Fig. 4A and B). In addition, VLY(P480W) had a substantial reduction in hemolytic efficiency on human erythrocytes (Fig. 4C). Sheep erythrocytes remained resistant to VLY(P480W) (Fig. 4C). Thus, P480 is necessary for efficient pore formation and cell activation by VLY, and VLY(P480W) may be useful as a genetic toxoid. In order to investigate further the potential role of this residue, we made the converse mutation at the corresponding location (W435P) in PLY, the species-nonspecific CDC from *S. pneumoniae*. This PLY mutant lysed erythrocytes, but only at concentrations much higher than those of wild-type toxin (Fig. 4D).

DISCUSSION

BV, a chronic infectious/inflammatory disease associated with preterm birth, is strongly linked with the mucosal overgrowth of *G. vaginalis* and its attachment to epithelial cells. However, mechanistic studies of BV have been hampered by

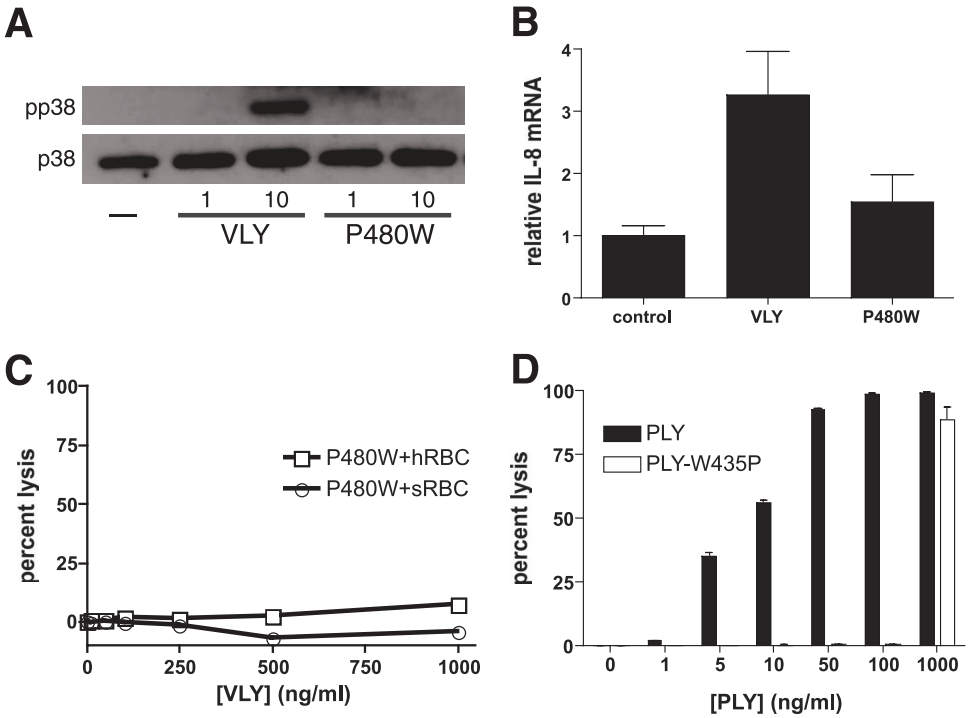

FIG. 4. VLY-mediated epithelial cell activation and erythrocyte lysis require P480. (A) Human cervical epithelial cell line HeLa was treated for 30 min with medium alone (−), VLY, or VLY(P480W) (1 to 10 μg/ml) prior to lysis and Western blotting with antibodies specific for total (p38) and phospho-p38 (pp38) MAPK. (B) HeLa cells were treated with VLY or VLY(P480W) (10 μg/ml) for 2 h prior to RNA extraction and assay of relative quantity of IL-8 message by real-time PCR. (C) Human (hRBC) and sheep (sRBC) erythrocytes were treated with the indicated concentrations of VLY or VLY(P480W), and hemolysis was assessed as described above. (D) Human (hRBC) erythrocytes were treated with the indicated concentrations of PLY or PLY(W435P), and hemolysis was assessed as described above.

the lack of an animal model. Attempts have been made to induce vaginitis with *G. vaginalis* in mice, rabbits, and nonhuman primates with only limited success (5). In contrast, early studies by Criswell et al. found that *G. vaginalis* in pure culture could cause vaginitis in human volunteers (9). We have cloned, sequenced, and characterized VLY, the human-specific CDC from *G. vaginalis*. VLY is the only known species-specific factor of *G. vaginalis* and represents a candidate virulence and host range determinant that should aid investigations of pathogenesis and further the development of specific diagnostic tests. Our findings, including activation of the p38 MAPK and IL-8 pathways in human epithelial cells, suggest that VLY produced by *G. vaginalis* may be a major factor in the immunopathology of BV. It is notable that dense neutrophilic infiltrates and high mucosal IL-8 are not generally associated with the clinical syndrome of BV, despite a large organism burden (7). This has been hypothesized to be the result of other virulence factors from *G. vaginalis* (such as its sialidase and prolidase) or from other resident organisms, leading to relatively low IL-8 and high IL-1β production (6).

VLY expands the CDC family to another gram-positive genus and to a novel anatomic site. CDCs are produced by organisms that colonize and cause disease at mucosal surfaces including the upper and lower respiratory tracts and the gastrointestinal tract. In many such cases, toxin production has been shown to be essential for maintenance of colonization, pathogenesis of invasive disease, or both (38). CDCs have been described only in gram-positive organisms. Thus, the characterization of VLY and its evolutionary relationship to the other CDCs provides further evidence that *G. vaginalis* is most properly grouped with the gram-positive bacteria, despite its variable staining characteristics (36).

The phylogram of CDC protein sequences (Fig. 1A) demonstrates three distinct groupings—a *Streptococcus* group (into which VLY also falls), a *Listeria* group, and a *Bacillus*/*Clostridium* group (also containing streptolysin O). The members of the *Streptococcus* clade have the most divergence in the domain 4 undecapeptide, including the presence of a proline residue as an insertion (pyolysin) or substitution (ILY and VLY). In the case of pyolysin, the unusual undecapeptide has been shown to be required for pore formation (3). Seeligeriolysin, the CDC from *Listeria seeligeri*, has an alanine-to-phenylalanine mutation in the undecapeptide that causes a decrease in toxin efficacy compared to that of listeriolysin O (21). Because of the importance of the undecapeptide to toxin function, we created VLY(P480W) in order to restore the consensus tryptophan residue. This mutant acted as a toxoid, lacking substantial lysis of human or sheep erythrocytes. Construction of the corresponding mutation in PLY led to a substantial decrease in its lytic activity. These findings emphasize the importance of the structure of the undecapeptide region to the function of CDCs. Likewise, the substitution of a lysine residue for the conserved cysteine in the undecapeptide is a modification unique to VLY. Prior reports have demonstrated that the *G. vaginalis* hemolysin is not thiol activated (35). The lack of enhancing effect of a reducing agent is consistent with this

modification in the undecapeptide. Of note, in other CDC family members, the conserved cysteine residue confers thiol-activating properties but is not essential for pore-forming activity (37).

The human specificity of the *G. vaginalis* hemolysin was noted in earlier studies (8), but no specific mechanism for this was described. The recent characterization of human CD59 as a receptor for ILY represented a major step forward in the understanding of the mechanism of action of CDCs, which were previously thought to bind cholesterol directly as the sole requirement for pore formation. It is now clear that this model is oversimplified and that at least a subset of the CDCs require protein receptors on the surface of target cells (15). The role of cholesterol appears to be complex, as preincubation of the CD59-dependent cytolysins VLY (this study) and ILY (31) with cholesterol can inhibit lytic activity and is thought to affect the prepore-to-pore transition. Domain swap experiments have indicated that the region dictating species-specific CD59 binding by ILY resides in domain 4, outside of the undecapeptide (15, 28). Alignment of VLY, ILY, and three species-nonspecific CDCs did not reveal obvious candidate amino acids in domain 4 that might confer human CD59 binding (see the supplemental material). It is particularly striking that CD59, a glycosylphosphatidylinositol-anchored protein that inhibits assembly of membrane attack complex components in a species-specific manner, should act as a toxin receptor. The description of a common structural fold essential for function of both CDCs and members of the membrane attack complex (17, 34) may lead to an improved mechanistic understanding of both of these interactions.

VLY represents the second species-specific, CD59-dependent member of the CDCs and should aid further mechanistic studies of this toxin family significantly. The requirement for human CD59 in VLY-induced cytolysis suggests a possible reason for the absence of a reproducible nonhuman model of disease. We suggest that either *G. vaginalis* expressing a species-nonspecific derivative of VLY or transgenic animals expressing human CD59 might be a useful model for the study of BV and its sequelae.

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