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Cytotoxicity of the *Vibrio vulnificus* MARTX toxin Effector DUF5 is linked to the C2A Subdomain

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Abstract

The multifunctional-autoprocessing repeats-in-toxin (MARTX) toxins bacterial protein toxins that serve as delivery platforms for cytotoxic effector domains. The domain of unknown function in position 5 (DUF5) effector domain is present in at least six different species' MARTX toxins and as a hypothetical protein in *Photobacterium* spp. Its presence increases the potency of the *Vibrio vulnificus* MARTX toxin in mouse virulence studies, indicating DUF5 directly contributes to pathogenesis. In this work, DUF5 is shown to be cytotoxic when transiently expressed in HeLa cells. DUF5 localized to the plasma membrane dependent upon its C1 domain and the cells become rounded dependent upon its C2 domain. Both full-length DUF5 and the C2 domain caused growth inhibition when expressed in *Saccharomyces cerevisiae*. A structural model of DUF5 was generated based on the structure of *Pasteurella multocida* toxin facilitating localization of the cytotoxic activity to a 186 amino acid subdomain termed C2A. Within this subdomain, an alanine scanning mutagenesis revealed aspartate-3721 and arginine-3841 as residues critical for cytotoxicity. These residues were also essential for HeLa cell intoxication when purified DUF5 fused to anthrax toxin lethal factor was delivered cytosolically. Thermal shift experiments indicated that these conserved residues are important to maintain protein structure, rather than for catalysis. The *Aeromonas hydrophila* MARTX toxin DUF5_{Ah} domain was also cytotoxic, while the weakly conserved C1-C2 domains from *P. multocida* toxin were not. Overall, this study is the first demonstration that DUF5 as found in MARTX toxins has cytotoxic activity that depends on conserved residues in the C2A subdomain.

Keywords

MARTX; *Vibrio vulnificus*; effector domains; yeast screen; cytotoxin

INTRODUCTION

Multifunctional-autoprocessing repeats-in-toxins (MARTX) toxins are large protein toxins (3500-5300 aa) secreted by Gram-negative bacteria¹. These toxins carry from 1 to 5 protein effector domains, but also function as a delivery platform for transfer of these effector

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domains across the eukaryotic cell plasma membrane. These domains are then excised from the holotoxin by autoprocessing and released to the eukaryotic cell cytosol²⁻⁴ where they function as “effectors” freed from the translocation system of the toxin²⁻⁴. Among the various MARTX toxins of different mammalian, aquatic, and insect pathogens, a total of 10 different effector domains are carried by MARTX toxins, although the number and positional organization of the arrayed effectors vary across strains and species¹. The effector domain repertoire of the toxins can be exchanged by uptake of exogenous DNA and incorporation of the new sequences and/or loss of old sequences by homologous recombination resulting in novel toxins in different strains of the same species^{5,6}.

Within the target cell, the effector domains are thought to each have cytopathic or cytotoxic activity such that the overall role of the toxin in the eukaryotic cell is the sum of the activities of the effectors it delivers. Thus, it is important to individually characterize the function of each effector using genetics, biochemistry, and cell biology approaches to understand how an effector exchange will affect bacterial pathogenesis.

Among the 10 MARTX effector domains identified by sequence comparisons, only three have been functionally characterized¹. The actin crosslinking domain (ACD) covalently links actin monomers via an isopeptide bond leading to actin cytoskeletal destruction⁷⁻¹⁰. The Rho GTPase inactivation domain (RID) disables the Rho regulatory pathway resulting in loss of active Rho and thereby to cytoskeleton depolymerization^{11,12}. The ExoY domain is an adenylate cyclase¹³. The remaining seven MARTX toxin effector domains are uncharacterized but are often similar to domains of other large protein toxins¹.

One of the domains of unknown function is known as DUF5, indicating its presence in the 5th effector domain position of the *Vibrio vulnificus* strain CMCP6 MARTX toxin where it was first recognized¹⁴ (holotoxin diagrammed in Fig. 1A). Within *V. vulnificus*, the presence of DUF5_{Vv} increases the potency of the toxin during mouse infection resulting in a lower LD₅₀ compared to an isogenic strain from which the effector domain was deleted or a naturally occurring strain that lost DUF5_{Vv} via a homologous recombination event⁵. Thus, DUF5_{Vv} is a virulence factor that increases the pathogenicity of the strains that carry it as a domain within the MARTX toxin.

DUF5_{Vv} was initially recognized to have sequence similarity to *Pasteurella multocida* toxin (PMT), whose carboxyl-terminus is composed of three domains: C1, C2, and C3¹⁵. The C1_{Pm} subdomain from PMT is known to be a four helical bundled membrane localization domain (4HBM)¹⁶. The conserved C1_{Vv} subdomain from DUF5_{Vv} has also been demonstrated to localize to the eukaryotic plasma cell membrane, where it binds anionic lipids via a basic-hydrophobic motif^{12,17}. Structural determination by nuclear magnetic resonance of the isolated C1_{Pm} and C1_{Vv} subdomains confirm both of these domains form a four helical bundle in solution^{18,19}.

However, none of the extensive characterization of PMT has revealed the function of its C2 domain. The PMT C3 domain is a deamidase enzyme with a catalytic cysteine residue that acts on the Gα subunits of trimeric G proteins²⁰⁻²³. It is notable that the sequence similarity of DUF5_{Vv} with PMT is limited to the C1 and C2 domains and DUF5_{Vv} does not share the

C3 deamidase domain and thus DUF5_{VV} is not expected to have a similar activity (Fig. 1A). DUF5 is present also within MARTX toxins of *Aeromonas hydrophila*, *Yersinia kristensenii*, *Vibrio splendidus*, and *Xenorhabdus nemotophila*¹ and as the stand-alone hypothetical protein *plu2400* in *Photobacterium* sp.,²⁴ where it might be an effector with a distinct delivery mechanism such as Type III secretion or the Tc complex²⁵.

In this study, we initiated a *de novo* investigation on this protein of unknown function. We generated a structural model of DUF5_{VV} based on the structure of the PMT C-terminus¹⁵. We then show that ectopic expression of the domain in HeLa cells is cytotoxic. In *Saccharomyces cerevisiae*, expression of the DUF5_{VV} causes growth inhibition. The toxic effect in HeLa cells is mapped to a 186 amino acid C2A subdomain and shown to require an Asp and Arg residue. Overall, these studies mark our initial efforts to establish that DUF5 is a *bona fide* MARTX toxin effector.

MATERIALS AND METHODS

Cell lines, media, reagents and plasmids

HeLa epithelial cells were grown at 37°C with 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies Gibco) with 10% fetal calf serum (Gemini Bio-Products, West Sacramento, CA), 100 U/ml penicillin, and 1 µg/ml streptomycin. J774 macrophages, COS7 fibroblasts, and HEp-2 epithelial cells were grown in identical conditions. *E. coli* DH5α, TOP10 (Life Technologies Invitrogen) and BL21(DE3) were grown at 37°C in Luria-Bertani (LB) liquid or agar medium containing either 100 µg/ml ampicillin or 50 µg/ml kanamycin as needed. *S. cerevisiae* strain InvSc1 (Invitrogen) was grown on YPD liquid or agar medium at 30°C or commercial synthetic complete (SC -ura) supplemented with yeast nitrogen base (MP Biomedicals) as detailed below. Media components and common reagents were obtained from Sigma-Aldrich, Fisher, or VWR and common restriction enzymes and polymerases from New England Biolabs or Invitrogen. Custom DNA oligonucleotides and gBlocks were purchased from Integrated DNA Technologies (Coralville, IA). Plasmids were prepared either by alkaline lysis with precipitation in ethanol or purified using Epoch spin columns according to manufacturer's recommended protocol.

Alignments and structural modeling

Proteins with homology to DUF5_{VV} from strain CMCP6 were identified using BLASTP²⁶ at the National Center for Biotechnology Information website. Amino acid sequences were trimmed to DUF5_{VV} homology region and aligned with CLUSTALW using MacVector 12.6.0. The DUF5_{VV} and DUF5_{VV} D3721A protein sequences were also aligned to the pdb database using HHpred²⁷ and a pdb structural model built based on published PMT structure (pdb 2EBF¹⁵) using Modeller²⁸. Figures were generated from the structural model using MacPyMol.

Construction of plasmids for ectopic expression in HeLa cells and yeast

DNA corresponding to coding sequence for amino acids 3579-4089 of the *V. vulnificus* *rtxA1* gene (GI: 27366913; VV2_0479) was amplified from purified *V. vulnificus* CMCP6

chromosomal DNA using Pfx50 DNA polymerase (Invitrogen) and primers 1 and 2 (5'-gagctagcatgggtgataaaaccaaggtcgtggattta and 5'-gccgtcgaccaaactgcccttgaacgtgatcttcggttt). The insert was digested with enzymes NheI and SalI and ligated into the similarly digested vector. For expression of the *P. multocida toxA* gene sequence corresponding to aa 573-1113 of PMT from strain 4533 (GI:149228008), a synthetic codon optimized double stranded DNA sequence was obtained from GenScript (Piscataway, NJ) in pUC57 and subcloned into pEGFP-N3 via the HindIII and BamHI sites.

DNA sequences above were similarly amplified except with novel EcoRI and KpnI restriction sites incorporated into the oligonucleotides for transfer into yeast expression vector pYC2 NT/A (Invitrogen) using primers 6,7,8 and 11 (5'-aaggtaccgtttatcggtgaagatgcaagttgcc, 5'-agaattctcacaactgcccttgaacgtgatc, 5'-aaggtaccgggtgataaaaccaaggtcgtg, and 5'-aaggtaccggatattgacgcttgggatcgt).

Ectopic expression of EGFP fusion proteins in HeLa cells

Plasmids for transfection were prepared using the Qiagen Midi Prep kit. HeLa cells grown to approximately 80% confluency were transfected using Eugene HD (Promega) and 2 µg plasmid DNA at a 4:1 ratio for 5 h after which fresh media was exchanged.

For western blotting, cells were collected 24 h after transfection in 2x SDS-PAGE buffer, boiled for 5 min, and the proteins were separated by SDS-PAGE and transferred to nitrocellulose by the tank blot method. Western blotting was done as previously described¹⁷ using anti-GFP antibody conjugated to horseradish peroxidase (Mileny Biotec 130-091-833) at a 1:1000 dilution. SuperSignal West Pico Chemiluminescent Substrate and autoradiography was used for detection.

For microscopy, HeLa cells were grown in 35 mm MatTek glass bottom microwell dishes to approximately 60% confluency and transfected as described above. Live cells were imaged 24 h after transfection by epifluorescence and differential interference contrast (DIC) microscopy at 200x using the Andor Spinning Disk confocal microscope. Images were overlaid using NIH ImageJ64 and assembled into figures using Adobe Photoshop CS6. Rounded cells were manually counted from at least 3 different transfections. Histograms of representative cells were plotted using GraphPad Prism 4.0 or 6.0.

Purification of proteins fused to anthrax toxin lethal factor N-terminus

Plasmid vector pRT24 is a variant of pABII²⁹ in which the coding sequence for amino acids 1-254 of anthrax toxin lethal factor (LF_N) are expressed with an N-terminal His-tag under control of the T7 promoter. The plasmid was modified to replace the single BamHI cloning site with an oligonucleotide that introduces the TEV cleavage site and ligation independent cloning site from pMCSG7³⁰. DUF5_V DNA sequences were amplified with primers 3 and 4 (5'-tacttccaatccaatgctgataaaaccaaggtcgtggatgattta and 5'-ttatccaatgtgaaagagcgggtatttgcgcactcaa) and integrated into pRT24 by ligation-independent cloning³⁰. A stop codon after the codon for Thr3765 was introduced to generate a sequence that would be truncated after C2A. Site-directed mutagenesis was then used to alter codons D3721 and R3841 to Ala as described above. DUF5 from *A. hydrophila* fused to LF_N (LF_N-

DUF5_{Ah}) was generated in the same manner as LF_N-DUF5_{Vv} except using primers 14 and 15 (5'-tactccaatccaatgctccgggcaaacggtggtgacg, and 5'-ttatccactccaatgctagacatcggcgtactcgacccgc) to amplify the sequence corresponding to the MARTX toxin aa 3069-3570 (GI: 117618727) from chromosomal DNA prepared from *A. hydrophila* 7966 obtained from the American Type Culture Collection.

LF_N and LF_N fusion proteins were expressed in *E. coli* BL21 (DE3). Briefly, overnight cultures were diluted 1:100 in fresh LB containing the 100 µg/ml ampicillin and grown to OD₆₀₀ = 1.0 at 37°C before inducing the cultures with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h at 32°C, except for LF_NDUF5_{Vv} expression which was induced with 0.5 mM IPTG and cells were grown at 30°C. *E. coli* cells were harvested by centrifugation at 10,100xg and resuspended in 100ml Urea Buffer A (500 mM NaCl, 20 mM Tris pH 7.4, 5 mM imidazole and 8 M urea with addition of protease inhibitor tablets (cOmplete, EDTA-free purchased Roche Applied Sciences). Resuspended cells were sonicated using a Branson Sonifier model 102C for 7 min at 50% amplitude with the standard disruptor. Crude lysates were centrifuged at 17,600xg for 30 min to remove particulates and the remainder of the lysate was filtered across a PALL Acrodisc 0.45 µ syringe filter. Lysate was loaded onto a 1 ml GE Healthcare HisTrap column using the ÄKTA purifier protein purification system (GE Healthcare). Column was washed with 5 ml Urea Buffer A with 10 mM imidazole, followed by 5 ml 50 mM imidazole buffer to remove contaminating proteins. His-tagged LF_N proteins were eluted using an imidazole gradient from 50 to 250 mM. Peak fractions corresponding to the protein of interest were collected, pooled, and dialyzed to remove imidazole into a buffer containing 500 mM NaCl, 20 mM Tris, and 2 M urea, pH 7.4. Proteins were further purified by gel exclusion chromatography in the same buffer using a 16 × 100 Superdex 200 column (GE Healthcare). Purified proteins were concentrated using Millipore Amicon Ultra 30K spin concentrators and glycerol was added so that the final buffer was 300 mM NaCl 12 mM Tris pH 7.4, 1.2M urea, 20% glycerol. Protein concentration was determined using the NanoDrop ND1000, and purity was estimated using SDS-PAGE. Proteins were stored at -80°C until used.

Protective antigen (PA) was purified from the soluble fraction of *E. coli* BL21(DE3). Cells were grown at 37°C to OD₆₀₀ = 0.8, then the culture was induced with 1 mM IPTG for 4 h at 30°C. Bacterial culture was harvested by centrifugation, then resuspended in 500 mM NaCl, 20 mM Tris, 5 mM imidazole, pH 8.0. Lysate was prepared as for LF_N fusion proteins above except buffers did not contain urea. Sizing was performed as described above in 500 mM NaCl, 20 mM Tris pH 8.0 buffer.

Intoxication of mammalian cells with LF_N fusion proteins and PA

All cell types were grown in 24 well tissue culture treated dishes (Falcon). 7 nM PA and 3 nM LF_N-fusion proteins were added to 1 ml culture media overlaying the cells. Cells were incubated for 24 or 48 h at 37°C in 5% CO₂, after which cells were imaged at 100x by phase microscopy using a Nikon CoolPix 995 digital camera affixed to a Nikon TS Eclipse 100 microscope. For quantification, rounded cells were manually counted representing at least 3 independent experiments and results were graphed as histograms using GraphPad Prism 4.0 or 6.0.

Assay for cell lysis

Lactate dehydrogenase (LDH) release from intoxicated cells was determined using the Cytotox 96 Non-Radioactive Cytotoxicity Assay (Promega). After intoxication, 50 µl of culture media was removed from each well, mixed with 50 µl of reaction reagent, and incubated at room temperature protected from light for 30 min. Upon addition of stop solution, absorbance was measured at 490 nm. For determination of total LDH, cells from the same wells were lysed by addition of Triton X-100 to the residual media to a final concentration of 0.1% and then sampled and assayed as described above to determine the maximum lysis value for each well. Percent cell lysis was calculated using the formula

$$\left(\frac{A490 \text{ media}}{A490 \text{ media} + \text{cells}} \right)_{\text{exp}} - \left(\frac{A490 \text{ media}}{A490 \text{ media} + \text{cells}} \right)_{\text{untreated}} * 100.$$

Assessment of yeast growth inhibition

S. cerevisiae strain InvSc1 was grown in YPD broth prior to transformation. Yeast cells were transformed using a PLATE solution method and transformants selected using SC agar medium without uracil, supplemented with glucose as previously described³¹.

Transformed yeast cells were inoculated into liquid glucose synthetic complete medium (without uracil) and grown overnight at 30°C. The next day, cultures were centrifuged and washed three times with sterile water. Each sample was resuspended in water and OD₆₀₀ was measured for each using Beckman Coulter DU530 Spectrophotometer. All samples were normalized to OD₆₀₀ = 0.5 and then were 10-fold serially diluted. 5 µl of each dilution was spotted on solid agar selective medium (-uracil) with either 20 mg/ml glucose or 20 mg/ml galactose and 10 mg/ml raffinose. The plates were incubated at 30°C for 3 days before growth was assessed and plates photographed using a digital camera.

For growth cures, OD₆₀₀ of overnight cultures was measured and inoculi were normalized to each other and then diluted into 50 ml of SC medium containing 20 mg/ml galactose and 10 mg/ml raffinose (-uracil) to induce expression from the plasmid. OD₆₀₀ was measured every 2 h for 12 h to document growth patterns.

Alanine scanning mutagenesis

Site-directed mutagenesis to introduce an alanine or stop codon at locations noted in text was carried out using PfuTurbo DNA polymerase (Invitrogen) and custom oligonucleotides designed via Agilent PrimerDesign software. After amplification, DNA was treated with DpnI and transformed to *E. coli* TOP10. Isolated plasmids were sequenced to confirm gain of the desired mutation and to check for absence of unintended mutations during DNA amplification. Double mutant D3721R/R3841D in pYC-DUF5 plasmid was generated by cohesive end cloning of a synthetic DNA gBlock containing the R3841D mutation in exchange for the wild type sequence via flanking BamHI and AatII restriction enzyme sites (5'-

atctttatggtcgcgattgaagaagccaacggtaaacacgtaggtttgacggacatgatggttcgttgggccaatgaagaaccatacttggcaccgaagcatggttacaaggcgaaacgccaagtacgttggtttgatgcgaagtaccacgtagatctaggtgagc).

Purification of recombinant 6xHis-tagged proteins for fluorescence thermal shift assays

DNA corresponding to DUF5_{VV} was inserted into the overexpression vector pMSCG7 by ligation independent cloning using primers 12 and 13, (5'-tacttccaatccaatgctcaagagctgaaagaaagagcaaaag and tacttccaatccaatgctcaagagctgaaagaaagagcaaaag). Additional mutations were generated by site directed mutagenesis. Plasmids were transformed into *E. coli* BL21 (DE3) for purification. Cells were grown to OD₆₀₀ = 0.8 at 37°C. The temperature was reduced to 18°C and protein expression induced by the addition of IPTG to a final concentration of 1 mM. Cells were grown overnight with shaking and then harvested by centrifugation. Bacteria were resuspended in a buffer containing 50 mM Tris (pH 8.3), 500 mM NaCl, 0.1% Triton X-100, and 5 mM β-mercaptoethanol and lysed by sonication. After centrifugation at 30,000xg for 30 min, the soluble lysate was filtered through a 0.22μm membrane and loaded onto a 1 ml HisTrap column using the ÄKTA purifier protein purification system (GE Healthcare). After washes with 50 mM Tris, 500 mM NaCl, 50 mM Imidazole pH 8.3, the proteins were eluted in the same buffer with 500 mM imidazole. Proteins were further purified by gel filtration chromatography (Superdex 75 (16/60), GE Healthcare) in buffer containing 10mM Tris-HCl, 500 mM NaCl, 5 mM β-mercaptoethanol, pH 8.3.

Fluorescence thermal shift assay

The experiment was performed using a 96-well thin-wall PCR plate (Axigen). 20 μl reactions consisted of 2 μM protein in a solution of 5x SYPRO orange dye (Life Technologies), 0.1 mM HEPES, 150 mM NaCl, pH 7.5. Fluorescence intensity was monitored using the StepOnePlus™ Real-Time PCR Systems (Life Technologies) instrument. Samples were heated from 25°C to 95°C at a scan rate of 1°C/min. T_m values were extrapolated using Protein Thermal Shift™ Assay software (Life Technologies).

RESULTS

DUF5_{VV}, but not C1C2_{PM}, is cytotoxic when ectopically expressed in HeLa cells

To determine if DUF5 is a *bona fide* effector with cytotoxic effects on cells, the DNA sequence corresponding to *V. vulnificus* aa 3579-4089 (DUF5_{VV}) was amplified and cloned into ectopic expression vector pEGFP-N3 for expression of DUF5_{VV} as a fusion to EGFP under control of the CMV promoter. The plasmid was transformed into cultured HeLa cervical carcinoma epithelial cells and EGFP-positive cells were imaged after 24 hr. Cells expressing EGFP had a normal, cuboidal shape with less than 8% of cells rounded (Fig. 1B). By contrast, 82% of cells ectopically expressing the DUF5_{VV}-EGFP fusion were small and rounded and many of the cells showed signs of blebbing indicating necrosis (Fig. 1B,D). Some cells that had not yet fully rounded or necrosed showed DUF5_{VV}-EGFP localized to the cell periphery, consistent with the presence of the C1 plasma membrane localization domain (Fig. 1C). Western blot detection of the DUF5_{VV}-EGFP fusion showed less total protein than detected for the EGFP-expressing control cells (Fig 1H), indicating that expression of this fusion protein was toxic to cells and many cells expressing the DUF5_{VV}-EGFP may have detached.

DUF5_{VV} has 24% sequence identity with the C1-C2 domains of PMT (C1C2_{Pm}) (Fig. 1A). Since the *toxA* gene is carried on a bacteriophage with a low GC content (35% GC), a eukaryotic codon-optimized, synthetic copy of *toxA* sequences corresponding to C1C2_{Pm} was obtained and expressed in cells generating a protein similar in size to DUF5_{VV}-EGFP (Fig. 1H). Cells expressing C1C2_{Pm}-EGFP appeared similar to EGFP-control expressing cells (Fig. 1F). These results support previous data^{20,21,32,33} that all toxic activities of PMT are due to the C3 deamidase domain that is absent in DUF5_{VV}. Further, these data show that the cytotoxic activity of DUF5_{VV} may not be conserved in C1C2_{Pm}, at least in HeLa cells.

Cytotoxicity of DUF5_{VV} in HeLa cells is linked to the C2A domain

Despite the absence of functional conservation, C1C2_{Pm} and DUF5_{VV} may share structural conservation, although the function of the domains diverged. A structural model of DUF5_{VV} was generated based on the PMT structures¹⁵. Based on this model, the amino acids of DUF5_{VV} corresponding to the C1_{VV} and C2_{VV} domain were identified. Upon deletion of gene sequences for the C1_{VV} subdomain, the C2_{VV}-EGFP fusion is no longer localized to the cell periphery. Those cells highly expressing C2_{VV}-EGFP appear rounded, while low expressing cells remained normal (Fig. 2D). These data are consistent with C2_{VV} being required for cytotoxicity and C1_{VV} being required for efficient delivery to the plasma membrane.

In addition, as shown also by two recent bioinformatics studies^{34,35}, the structural model showed that C2_{VV} could be split into two subdomains, C2A_{VV} and C2B_{VV} (Fig 2A). To determine if the cytotoxic activity of C2_{VV} is linked its C2A or C2B subdomain, DNA corresponding to the individual subdomains was cloned fused to *egfp* and expressed in HeLa cells. Cells ectopically expressing only C2A_{VV}-EGFP were highly necrotic, while cells expressing C2B alone appeared normal (Fig. 3B–G) and produced EGFP-fusion protein detectable by western blotting (Fig. 3H). However, due to the severe toxicity of C2A alone resulting in poor sample recovery, a corresponding fusion protein could not be detected by western blotting to confirm expression (Fig. 3H).

As an alternative verification of the cytotoxicity associated with C2A_{VV}, both full-length DUF5_{VV} and C1-C2A from *V. vulnificus* were purified fused to His-tagged *B. anthracis* LF_N that is often used as a bioporter for toxin effectors in the absence of the holotoxin^{7,11,29,36}. The purified proteins were insoluble in less than 2M urea, but nevertheless retained toxicity after delivery to cells by PA. The snap dilution out of urea in the tissue culture media likely allowed folding of the LF_N domain and the protein then associated with PA for translocation and successful refolding of the DUF5_{VV} domain within the cytosol. Notably, both the full-length protein (Fig. 3J) and the C1-C2A fragment (Fig. 3K) resulted in rounding of cells confirming transfection studies that C2A is sufficient for cytotoxicity of DUF5_{VV} in HeLa cells. Furthermore, LF_NDUF5_{VV} was cytotoxic to other mammalian cell types as well, including J774 macrophages, COS7 fibroblasts, and HEp-2 epithelial cells (Table I).

DUF5_{Ah} from *A. hydrophila* is also cytotoxic

As shown in Fig. 5, proteins similar to DUF5_{VV} and PMT C1-C2 subdomains are found as uncharacterized proteins from other bacterial species. To further explore the possibility that

these proteins comprise a novel functional family of cytotoxins, the DUF5-like effector domain from the *A. hydrophila* MARTX toxin was cloned in fusion with LF_N and delivered to HeLa cells via PA. Protein purity was assessed by SDS-PAGE in panel 4C. After intoxication it was observed that HeLa cells were rounded similarly to what is seen with LF_N-DUF5 (Fig. 4 A,B) indicating that this effector domain also has cytotoxic function. Furthermore, the rounding efficiency is similar between the two toxins (Fig. 4 D, E). Finally, neither toxin induced cell lysis when delivered to HeLa cells, at any of the concentrations tested (Fig. 4 F, G).

Both full-length DUF5_{VV} and C2 alone cause growth inhibition when expressed in yeast

To further explore the function of DUF5_{VV}, we tested if it would be toxic if expressed in *S. cerevisiae*. The gene sequence for DUF5_{VV} was cloned into the yeast expression vector pYC2/NTA placing the gene under the control of a galactose-inducible promoter. When transformed into yeast, the DUF5_{VV}-expressing yeast strain grew poorly under non-inducing conditions and showed no growth under inducing conditions on either plates or broth culture (Fig. 6A). Indeed, expression of DUF5_{VV} was more toxic than the MARTX ACD effector domain that has been previously studied in yeast (Fig. 6A)³¹. Toxicity was reduced by removal of the C1 MLD such that cells expressing C2 alone were viable under non-inducing conditions with 100- to 1000-fold reduced plating efficiency on galactose and no growth in broth culture (Fig. 6A). The C1 MLD alone is not toxic when expressed in yeast (Fig. 6A), as previously shown^{31,37}.

Distinct from studies in HeLa cells, yeast cells expressing C2A alone were viable when plated on galactose (Fig. 6B). As an alternative verification for the essentiality of C2B in yeast, stop codons were introduced in the yeast expression plasmid at the codons for V3906 and G3948. Similar to expression of C2A alone, cells expressing proteins truncated within C2B also grew under inducing conditions on both plates and in broth (Fig. 6C). Close examination of the plating efficiency of C2A compared to C2B indicates that expression of C2A alone may show a slight growth inhibition on plates or in broth but the effect is modest (Fig. 6B).

Overall, in yeast, distinct from HeLa cells, both C2A and C2B are required for full toxicity although some toxicity is exhibited by C2A alone. The additional requirement for C2B in yeast may reflect a modest difference in the stability of the protein in yeast.

Identification of a C2A inactivating mutation by alanine scanning mutagenesis in *S. cerevisiae*

Alanine scanning mutagenesis has proven to be a useful tool to identify critical residues for other of MARTX effector domains^{12,31,38}. Alignment of DUF5 amino acid sequences from 5 MARTX toxins, Plu2400, and PMT showed that there are only 16 residues (8.5%) that are 100% identical across all proteins. If the potentially inactive PMT is excluded, 38 residues (20%) are identical across the remaining 6 effectors (Fig. 5).

To avoid severe toxicity associated with expression of full-length DUF5_{VV} in yeast, the plasmid for expression of C2 without the MLD was modified by site-directed mutagenesis

targeting 65 total residues in C2A (Fig. 5, indicated by asterisks), focusing predominantly on polar residues known to be important for catalysis of other bacterial toxins. Both conserved and non-conserved residues were changed to alanine codons. In addition, nine highly conserved residues in C2B were changed for alanine.

S. cerevisiae transformed with mutagenized plasmids were recovered by growth on glucose and then tested for the ability to grow on galactose. Surprisingly, 73/74 of the mutations did not alter the growth inhibition exhibited by strains expressing unaltered C2. The high frequency of mutations showing no relief of toxicity suggests that modest changes to overall structure are not sufficient to overcome the severe toxicity of this protein for yeast, even when the C1 MLD is absent.

Only one mutant, D3721A found within the C2A domain was identified that facilitated growth of yeast expressing C2. By contrast, a more conservative substitution to glutamic acid did not restore growth to yeast (Fig. 7A). D3721 is one of the 16 residues within C2A that is highly conserved in all the DUF5-like proteins, including PMT (Fig. 5).

As an independent verification of the importance of this residue to the function DUF5_{VV}, the mutation was transferred onto the full-length clone of DUF5_{VV} for expression in yeast. In this background, the mutation improved growth of yeast under non-inducing conditions to levels near vector control. Under inducing conditions, the plating efficiency was improved 100- to 1000-fold compared to expression of full-length DUF5_{VV}, although the growth inhibition was not alleviated as shown in liquid culture experiments (Fig. 7B).

Examination of the structural model of DUF5_{VV} (Fig. 2A) showed that D3721 is present on helix 3 of C2A and makes polar contacts with R3841 on the final helix of C2A just before the start of C2B (Fig. 7C,D). Previous change of R3841 to Ala as part of the screen indicated this residue was not essential in the context of C2. However, in the context of the full-length DUF5_{VV} that includes the MLD, the phenotype of DUF5_{VV} R3841A is nearly identical to the phenotype of the D3721A resulting in an improved plating efficiency but poor growth in broth culture (Fig. 7B). This residue is also among the 16 100% identical residues found within C2A (Fig. 5). Combining the D3721A and R3841A resulted in a phenotype identical to that observed for either D3721A or R3841A substitutions alone and did not demonstrate an additive effect. Swapping the aspartate and arginine (D3721R/R3841D) did not improve growth either by plating or broth culture indicating that the potential bridge created by these residues is positional specific.

D3721 and R3841 are essential for cytotoxicity

As a final demonstration of the structural requirements for cytotoxicity, the D3721A and R3841A mutations were introduced onto the recombinant overexpression plasmid for production of C1-C2A fused to LF_N. Proteins were prepared for each mutant from insoluble pellets, urea was reduced to 1.2 M, and the unfolded proteins were delivered to cells by PA. Both mutants lost function in cytotoxicity compared to the similarly prepared unmodified LF_NC1-C2A_{VV} protein (Fig. 8A–E). Assessment of intoxication over time showed that cells treated with PA plus full-length LF_NDUF5_{VV} did not recover after 24 h intoxication and nearly 100% of cells remained rounded out to 48 hr. By contrast, ~50% of cells initially

intoxicated with PA plus LF_NC1-C2A_{VV} recovered between 24 and 48 h and returned to normal shape. These data suggest either that C2B carries an additional cytopathic function that prevents recovery of the rounded cells or, more likely, that C2B stabilizes C2A such that the toxin avoids turnover in the cells after successfully inducing cell intoxication. In support of this possibility, fluorescence thermal shift experiments were conducted with full-length recombinant 6xHis-DUF5_{VV} without fusion to LF_N (Fig. 7E) This recombinant 6xHis-DUF5_{VV} has a half-maximal melting temperature (T_m) of 43.8°C, while the D3271A substitution lowers T_m by 6.0°C to 37.8°C. The lower T_m indicates that D3271A causes a structural disturbance that can explain the reduced toxicity seen in yeast and HeLa cells indicating its interaction with R3841 may function to stabilize the protein structure rather than serve as a catalytic residue (Fig. 7F). This is also consistent with the structural model of DUF5 where D3721 is located within the core of the protein, such that a mutation to alanine would cause a disturbance consistent with a drop in T_m and would also account for the higher initial fluorescence seen with DUF5 D3721A than wild type protein.

DISCUSSION

In this study, we undertook a structure-function approach to discover if the *V. vulnificus* MARTX toxin effector domain DUF5_{VV} is a cytotoxin accounting for its dramatic effect on virulence in mouse infection studies⁵. The C1_{VV} subdomain of this protein has been previously shown to localize to anionic membranes, but the function of the C2_{VV} subdomain at the membrane had not been previously investigated. Here, we demonstrate that DUF5_{VV} effector domain is cytotoxic to HeLa cells and to yeast resulting in growth inhibition. Further, the cytotoxic activity is localized to its C2A subdomain. In retrospect, mapping the cytotoxicity to the C2A subdomain is surprising because recent computer-based modeling studies of DUF5_{VV} and related proteins linked the C2B domain to the TIKI/TraB family of proteases leading to the proposal that C2B is a peptidase that functions in signaling^{34,35}. However, we found that any putative protease activity associated with C2B would not contribute to cytotoxicity as complete removal of the subdomain from DUF5_{VV}-EGFP did not affect cytotoxicity after ectopic expression studies in HeLa cells and expression of C2B-EGFP did not cause any observable effect in HeLa cells. Further the computer-based analysis indicated that C2B residue H3902 would be essential for peptidase activity, but this residue was among those modified during expression in yeast that did not restore the ability of yeast to grow (Fig. 5). These findings convincingly link the cytotoxic effect of DUF5_{VV} to its C2A subdomain; however, we cannot exclude that the C2B in addition to C2A could modify cell biological processes in manner that does not affect cell viability or morphology during MARTX intoxication and that DUF5_{VV} itself is a multifunctional effector domain.

The remainder of the study focused on identification of residues within C2A_{VV} that are essential for its cytotoxicity. Growth of yeast expressing C2_{VV} was used as a method to screen point mutations to identify those that would overcome the severe toxicity in yeast, a highly stringent phenotype generally indicative of an essential residue. The screen revealed a single essential Asp that initially was considered as a possible catalytic residue. However, the absence of additional residues in C2A_{VV} that would be predicted to form a catalytic site along with the finding that other highly conserved Gly, Pro, Tyr, Phe, Leu, and Ala are not essential suggests this subdomain functions by binding to a target protein rather than by

covalent modification. The ability of the residue to tolerate substitution to the more structurally conservative glutamic acid also indicates this is not likely an aspartyl protease. We further found that the D3721A substitution reduced the T_m of the DUF5_{VV} indicating structural destabilization as opposed to loss of catalytic function.

This stabilization may be due to its association with R3841 to retain optimal folding of the face that binds to the target protein or to serve as a switch to facilitate a change in the DUF5_{VV} structural conformation upon binding of C1_{VV} to the membrane (Fig. 7D). The role as a switch in the context of membrane binding is particularly interesting since reduced toxicity due to R3841A was observed only in the context of the C1_{VV} membrane localization domain in both yeast and HeLa cells. The contact between D3721 and R3841 could affect the conformation at the interface between C2A_{VV} and C2B_{VV} since R3841 that makes polar contacts with D3721 also contacts a S3986 in an unstructured loop of C2B. In other DUF5 homologues, the Ser is replaced by a Thr. Further, this Ser is absent from PMT, although Ser residues are localized nearby in this otherwise poorly conserved regions between PMT and DUF5_{VV}. Thus, it is intriguing to speculate that C2B_{VV} could function as a stabilization subdomain for C2A_{VV} with D3721 and R3841 functioning as part of the conformational switch to open up a binding site for the cellular target of C2A (Fig. 9).

A final component not addressed in this study is the biochemical mechanism or activity of DUF5_{VV} and DUF5_{Ah}. While two residues, D3721 and R3841 were found to be essential for rounding of mammalian cells by DUF5_{VV}, this discovery does not as yet inform the biochemical or cell biological activity that results in cell rounding. This is particularly true since residues shown to be essential for DUF5_{VV} (D3721 and R3841) and conserved in DUF5_{Ah} (D3215 and R33e5) are also conserved in PMT (as D720 and R861). Given that PMT is not able to round cells similar to DUF5_{VV} and DUF5_{Ah}, we can only speculate that surrounding residues not conserved in PMT also contribute to the appropriate structure for DUF5_{VV} and DUF5_{Ah} allowing these proteins but not PMT to properly interact with cellular components. Despite not yet directly demonstrating the biochemical or cell biological activity of the MARTX DUF5 effector domains, this study has provided numerous useful tools and reagents for these on-going studies but likewise reveals how identification of the cellular target could potentially be problematic. We found that the cytotoxicity is associated with C2A. However, this subdomain is highly toxic when ectopically overexpressed, which presents difficulties in identifying the target protein by common affinity precipitation techniques. A catalytically inactive variant is often highly useful to trap targets by affinity precipitation methods, but we found that the only inactive substitution also affects structural integrity and likely no longer binds its target in vivo. Our findings here that yeast is also affected by DUF5_{VV} does open the possibility that yeast-based genetic approaches could be very helpful to identify the target and these studies are currently ongoing.

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ABBREVIATIONS

aa	amino acids
ACD	actin crosslinking domain
BLASTP	basic local alignment search tool protein
DUF5	domain of unknown function 5
DIC	differential interference contrast
DMEM	Dulbecco's Modified Eagle's Medium
EGFP	eukaryotic green fluorescent protein
FTS	fluorescence thermal shift
IPTG	isopropylthio- β -galactoside
LF_N	lethal factor N-terminal portion
MARTX	multifunctional autoprocessing repeats-in-toxin
MLD	membrane localization domain
PA	protective antigen
PMT	<i>Pasteurella multocida</i> toxin
<i>sp.</i>	species
SC	synthetic complete
TIKI- TraB- RID	Rho inactivation domain
YPD	yeast peptone dextrose
4HBM	four helical bundle of the membrane localization domain

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FIGURE 1.

DUF5 from *V. vulnificus* MARTX toxin is cytotoxic to HeLa cells. **(A)** Scale drawing of *V. vulnificus* MARTX and *P. multocida* PMT protein toxins with enlarged region showing C1, C2A, and C2B domains that are shared between the two toxins. **(B–F)** Epifluorescent and DIC images (200x) of HeLa epithelial cells transfected with pEGFP-N3 plasmid clones expressing EGFP **(B)**, DUF5_{VV}-EGFP **(C–E)**, or C1C2_{Pm}-EGFP **(F)**. Panels **(D)** and **(E)** enlarged 200% to show detail of localization of DUF5_{VV}-EGFP and cell blebbing, respectively. Arrows in panel **E** indicate EGFP-positive cells in DIC only image. Percent of rounded cells in each cell type is quantified from three independent experiments **(G)** and expression of protein in transfected cells is shown by western blot detection using an anti-GFP antibody **(H)**.

FIGURE 2.

The C1 MLD of DUF5_{VV} is necessary only for efficient cell rounding. (A) Structural model of DUF5_{VV} generated in HHPRED and Modeller based on published structure of PMT. C1, C2A, and C2B subdomains are indicated. (B–D) Epifluorescent and DIC images (200x) of HeLa epithelial cells transfected with pEGFP-N3 plasmid clones expressing EGFP (B), DUF5_{VV}-EGFP (C), or C2-EGFP (E) and C2B-EGFP (F). Average of percent rounded cells in each cell type is quantified from three independent experiments (G) and expression of protein in transfected cells is shown by western blot detection using anti-GFP antibody (H). Note that C2A-EGFP could not be detected due to consistent poor sample recovery from plates due to toxicity of this domain. (I–J) DIC images of HeLa cells intoxicated with 7 nM PA in combination with 3 nM purified unmodified LF_N (I), LF_N fused to DUF5_{VV} (J) and LF_N fused to only the C1-C2A subdomains of DUF5_{VV} (K). LF_N-DUF5_{Ah} was also tested for ability to round cells as compared to LF_N control samples in panels (L) and (M). Protein purity was assessed via SDS-PAGE (N). Three independent experiments were performed and the number of round cells quantified in panel O.

FIGURE 3.

C2A is the cytotoxic subdomain of DUF5_{VV}. (**A**). Schematic of proteins expressed in the panel. (**B–H**) Epifluorescent and DIC images (200x) of HeLa epithelial cells transfected with pEGFP-N3 plasmid clones expressing EGFP (**B**), DUF5_{VV}-EGFP (**C**), C2-EGFP (**D**), C2A-EGFP (**E**) and C2B-EGFP (**F**). Average of percent rounded cells in each cell type is quantified from three independent experiments (**G**) and expression of protein in transfected cells is shown by western blot detection using anti-GFP antibody (**H**). Note that C2A-EGFP could not be detected due to consistent poor sample recovery from plates due to

toxicity of this domain. (**I–J**) DIC images of HeLa cells intoxicated with 7 nM PA in combination with 3 nM purified unmodified LF_N (**I**), LF_N fused to DUF5_{VV} (**J**) and LF_N fused to only the C1-C2A subdomains of DUF5_{VV}

FIGURE 4. DUF5 from *A. hydrophila*

is cytotoxic and causes cell rounding. LF_N-DUF5_{Ah} caused cell rounding when delivered to HeLa cells (A–B). Protein purity was assessed with SDS-PAGE (C). Rounding efficiency was comparable to DUF5_{Vv}, at all concentrations tested (D, E). Finally, release of LDH from intoxicated cells was measured (F, G), showing that there is no appreciable lysis when cells are intoxicated with either DUF5 protein.

FIGURE 5.

Amino acid alignment generated in MacVector 12.6.0 of only the C2A and C2B subdomains. Grey shading indicates 100% identical residues. Triangles indicate that sections of sequence were removed during alignment calculations. Asterisk indicated residues changed to alanine via site directed mutagenesis and boxes indicate two aa identified as important for growth inhibition in yeast. Larger asterisks indicate residues G3948 and V3906 which were mutated to stop codons, while the last large asterisk indicates S3986, which was not targeted in the initial mutagenesis but was later found by structural modeling to potentially interact with R3841.

FIGURE 6.

DUF5_{VV} and the C2 domain cause growth inhibition when expressed in yeast. *S. cerevisiae* strain InvSc1 was transformed with pYC2 NT/A plasmid expressing proteins indicated at left or with empty vector (EV), actin crosslinking domain from *V. cholerae* MARTX (ACD), or the C2 domain with stop codons introduced at V3906 or G3048. Panels show 5 μ l of 10-fold serial dilutions spotted to SC agar without uracil supplemented with either glucose (non-inducing) or galactose and raffinose (inducing). Panels at right show 12 h growth curves of cultures in SC broth with galactose.

FIGURE 7.

D3721 and R3841 are important residues for growth inhibition of yeast. Growth inhibition in yeast for C2-D3721A and C2-D3721E (**A**) and DUF5_V-D3721A, DUF5_V-R3841A, DUF5 with both residues mutated to alanine (DARA) and DUF5 with swapped residues (DRRD) in panel **B**. See figure legend 4 for details. (**C**, **D**) Structural model of DUF5_V showing polar contacts of D3721 and R3841 and potential cross association of R3841 with S3986 in C2B. Color-coding is the same as in Fig. 2A. Panel **E** shows the purified 6 x His-tagged DUF5 and DUF5 D3721A proteins that were used in FTS experiments to determine melting temperature in panel **F**.

FIGURE 8.

D3721 and R3841 are important residues for intoxication of HeLa cells. (A–E) DIC images of HeLa cells intoxicated for 24 h (upper) or 48 h (lower) with 7 nM PA in combination with 3 nM purified unmodified LF_N (A), LF_N fused to DUF5_{VV} (B) and LF_N fused to only the C1-C2A subdomains of DUF5_{VV} (C), or LF_N fused to only the C1-C2A subdomains of DUF5_{VV} with D3721 (D) or R3841 (E) point mutations. Protein purity was assessed by SDS-PAGE in panel F. Three independent experiments were performed and cells were manually counted (G).

FIGURE 9.

MARTX toxin undergoes autoprocessing upon entry into the host cell. Autoprocessing by the inositol hexakisphosphate bound cysteine protease domain releases other effector domains and allows them to perform their functions. DUF5 has been shown to be a stable protein when all the subdomains are present, and is able to efficiently round cells when the C2 domain is intact. When C2B subdomain is removed from the protein, leaving only C1-C2A, cell rounding is less efficient, presumably due to protein turnover. Therefore, C2B is hypothesized to be involved in stabilizing the interaction between DUF5 and the cellular target. C2A and C2B are required for a stable interaction with the target protein, but C2A alone is sufficient for cytotoxic activity.

Table ICell lines susceptible to DUF5_{VV} cytotoxicity^a

Cell Line	Rounding induced by LF _N DUF5 _{VV} ?	
	+ PA	– PA
HeLa human cervical carcinoma	+	–
COS7 African green monkey fibroblast	+	–
J774 murine macrophage	+	–
HEp-2 human laryngeal epithelial	+	–

^a Cells were intoxicated with LF_NDUF5_{VV} in the presence (+) or absence (–) of PA. After 24 hr, rounding was observed by phase microscopy. Intoxication conditions were the same as reported in figure legend 3.