Identification of a His-Asp-Cys Catalytic Triad Essential for Function of the Rho Inactivation Domain (RID) of Vibrio cholerae MARTX Toxin*

Background: The Vibrio cholerae MARTX toxin Rho inactivation domain (RIDVc) currently has an unknown mechanism of action. Residues His-2782, Leu-2851, Asp-2854, and Cys-3022 are shown to be essential for RIDVc activity. His-Asp-Cys form a catalytic triad necessary for enzymatic modification of the cellular target. RID effectors are Clan CE cysteine endopeptidases essential for Rho GTPase inactivation.

Results: Mutagenesis in the activity subdomain of RIDVc, four residues, His-2782, Leu-2851, Asp-2854, and Cys-3022, were identified as impacting RIDVc function in depolymerization of the actin cytoskeleton and inactivation of RhoA. Tyr-2807 and Tyr-3015 were identified as important potentially for forming the active structure for substrate contact but are not involved in catalysis or post translational modifications. Finally, V. cholerae strains modified to carry a catalytically inactive RIDVc show that the rate and efficiency of MARTXVc actin cross-linking activity does not depend on a functional RIDVc, demonstrating that these domains function independently in actin depolymerization. Overall, our results indicate a His-Asp-Cys catalytic triad is essential for function of the RID effector domain family shared by MARTX toxins produced by many Gram-negative bacteria.

Conclusion: In addition to the well studied major virulence factor cholera toxin that induces the severe diarrhea, V. cholerae secretes accessory toxins during the early stages of the infection that contribute to intestinal colonization by promoting evasion of innate immune cells including neutrophils and macrophages (1–3). Among these secreted accessory toxins is a multifunctional autoprocessing repeats-in-toxin (MARTX)5 toxin. MARTX toxins are large exotoxins of 350–560 kDa characterized by the presence of amino acid (aa) repeats at the N and C termini that are thought to form a pore-like structure necessary for translocation of the central portion of the toxin across the eukaryotic plasma cell membrane (4). The translocated portion of the toxin includes a cysteine protease domain (CPD) that is necessary for inositol hexakisphosphate-induced autoproteolysis after Leu residues in unstructured regions of the holotoxin to release effector domains into the host cell cytosol (5–8). Depending on the bacterial strain or species, a MARTX toxin carries an assortment of one to five effector domains that can be arrayed in different combinations and are exchanged between bacterial species by homologous recombination (4, 9, 10).

The MARTX of V. cholerae (MARTXVc) carries three effector domains (4). The actin cross-linking domain (ACDVc) is an effector domain that causes cell rounding by covalently cross-linking monomeric G-actin to form actin oligomers. It is related to the glutamine synthetase family of enzymes and irreversibly connects G-actin monomers via isopeptide bonds between residues Lys-50 and Glu-270 in the presence of ATP and Mg2+/Mn2+ (11–15). Another effector domain, the αβ-hydrolase domain, has been identified by analysis of CPD processing sites (7, 16) and by sequence homology to αβ-hydrolase family members (17), although its mechanism of action has not been assessed experimentally.

The third effector domain created by CPD autoprocessing after aa Leu-2433 and Leu-3085 (Fig. 1A) is the Rho inactivation

The diarrheal disease cholera caused by the Gram-negative bacterium Vibrio cholerae is characterized by massive efflux of water from intestinal enterocytes resulting in death by dehydration.

* This work was supported, in whole or in part, by National Institutes of Health Grant RO1 AI051490 (to K. J. F. S.). This work was also supported by Burroughs Wellcome Investigators in Pathogenesis of Infectious Diseases (to K. J. F. S.).

** This article contains supplemental Table S1.

1 Supported by the Howard Hughes Medical Institute International Student Research Fellows program.

2 Supported by an NRS A (National Research Service Award) Post Doctoral Research Fellowship F32AI075764.

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5 The abbreviations used are: MARTX, multifunctional autoprocessing repeats-in-toxin; RID, Rho inactivation domain; 4HBM, four helical bundle membrane localization domain; aa, amino acids; ACD, actin cross-linking domain; AD, activity subdomain of RIDVc; EGFP, eukaryote-optimized green fluorescence protein; GAP, GTPase-activating protein; CPD, cysteine protease domain; PA, protective antigen; LFν N terminus of lethal factor.
domain (RID\textsubscript{vc}) (7, 16), which induces a 90% decrease in cellular levels of active GTP-bound RhoA (RhoA\textsubscript{GTP}) as well as reduced activation of related GTPases Cdc42 and Rac, the net results of the loss of active RhoA GTPases is depolymerization of the actin cytoskeleton (18). The RID\textsubscript{vc} consists of two subdomains one of which is a four-helical bundle membrane localization domain (4HBM) that targets RID\textsubscript{vc} to the eukaryotic plasma membrane enriched at sites of cell-cell contacts (19, 20). This 4HBM has recently been shown to directly interact with the plasma membrane via a basic-hydrophobic motif composed of positively charged Lys-2577 and Arg-2630 in loops 1 and 3 of the bundle that interacts with negatively charged phospholipid head groups. Thr-2575 and Ser-2628 may also facilitate membrane interactions (19, 20).

The second subdomain of the RID\textsubscript{vc} from residues 2721–3085 is the activity subdomain (AD). Transient overexpression of the AD in epithelial cells has shown that this subdomain is necessary and sufficient to cause depolymerization of the actin cytoskeleton (20). In silico analysis of the AD predicts a circularly permuted papain-like thiol protease-fold suggesting RID\textsubscript{vc} could function as a cysteine protease or acyltransferase (21). However, this putative catalytic activity is not likely directed specifically against RhoA as the cell is fully restored upon removal of toxin-producing bacteria even when eukaryotic protein biosynthesis is blocked using cycloheximide (18). These results indicate that RhoA is neither irreversibly cleaved nor covalently modified. Thus, the direct target of the RID\textsubscript{vc} is likely a protein in the regulatory circuit that controls RhoGTPases activation (18).

In this study we perform site-directed mutagenesis to alter aa of the AD of RID\textsubscript{vc} to experimentally identify residues that are critical for actin depolymerization and RhoA inactivation in cultured epithelial cells. The analysis demonstrates that the function of RID\textsubscript{vc} depends on a His-Asp-Cys catalytic triad when overexpressed in cells by transient transfection, when transferred to cells directly, or when delivered to cells in the context of the MARTX\textsubscript{vc} holotoxin. In addition, conserved Tyr residues potentially contribute to substrate or cofactor binding but do not contribute to catalysis as previously predicted (21) or constitute sites of post-translational modifications. Finally, using V. cholerae carrying a MARTX toxin with an inactivated RID\textsubscript{vc}, we show that RID\textsubscript{vc} activity does not alter patterns or dynamics of actin cross-linking by ACD\textsubscript{vc} in intoxicated epithelial cells, suggesting that the increased availability of G-actin in the cell due to RID\textsubscript{vc} activity does not enhance the activity of the ACD\textsubscript{vc}.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Media, and Reagents**—Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown at 37 °C in Luria-Bertani (LB) liquid medium or agar plates containing 100 μg/ml ampicillin, 50 μg/ml kanamycin, or 50 μg/ml streptomycin as needed. HeLa cells were grown at 37 °C, 5% CO\textsubscript{2} in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) with 10% fetal bovine serum (FBS, Invitrogen), 50 μg/ml penicillin, and 50 μg/ml streptomycin.

**Site-directed Mutagenesis**—DNA sequence amplification of the RID\textsubscript{vc} corresponding to aa 2552–3099 and cloning into pEGFP-N3 to create pKS113 (pRID\textsubscript{vc}-EGFP) and bacterial expression plasmid pKS119 (pLF\textsubscript{vc}RID\textsubscript{vc}) was described previously (18). Site-directed mutagenesis on these plasmids were carried out using the Stratagene QuickChange II kit (Agilent, Clara, CA) according to the recommended protocol using primers as listed in supplemental Table S1. Plasmids were isolated by alkaline lysis and ethanol precipitation or according to the Qiagen Spin Miniprep kit (Qiagen, Valencia, CA) according to recommended protocol. Isolated plasmids were then submitted to DNA sequencing at the Northwestern University Genomics Core Facility.

**Transferring H2782A and C3022A Mutations onto the V. cholerae Chromosome**—The pKS113 DNA sequence corresponding to RID\textsubscript{vc} was modified by site-directed mutagenesis to introduce the H2782A or C3022A codon change in conjunction with a second modification to introduce a novel restriction site (SfoI and MluI, respectively). The modified DNA sequences were then amplified and cloned into the pCR-TOPO blunt vector according to the manufacturer’s protocol (Invitrogen). RID\textsubscript{vc}-mutants were subcloned into the SacB counter-selectable plasmid pWM91 using BamHI and XhoI generating plasmids pSA129 and pBLG21, and the SacB counter-selection method was used as described previously (22) to transfer the H2782A or C3022A mutations onto the chromosome of V. cholerae Δacd strain CC05 generating strains SAVH1 and BGV1, respectively, onto acd\textsuperscript{+} strain KFV119 generating SAVH2.

**Transient Transfection, Staining, and Microscopy**—HeLa cells were cultured for 24 h to ~50–70% confluency and then transfected using FuGENE\textsuperscript{®} HD (Roche Applied Sciences) or XtremeGene HP (Roche Applied Sciences) mixed with purified plasmid DNA at a ratio of 5:2 and 2:1, respectively, according to the manufacturer’s protocol. After 18–20 h at 37 °C, 5% CO\textsubscript{2}, cells seeded onto 18-mm circle coverslips were stained for microscopy with (1 unit) rhodamine phalloidin (Invitrogen) and 0.35 μM 4′,6′-diamino-2-phenylindole (DAPI) dihydrochloride (Invitrogen) as described previously (8). Stained cells were counted by fluorescence microscopy (Nikon Eclipse TS100) at a magnification of 400× and imaged by confocal microscopy at 1000× (LSM510 META-UV, Zeiss). For Western blots, cells grown in 6-well culture plates were lysed in 250 μl of 2× SDS-PAGE buffer and analyzed by using monoclonal anti-GFP antibody coupled to horseradish peroxidase (HRP) (1:1000, Miltenyi Biotec Inc., Auburn, CA) or polyclonal rabbit anti-actin antibody (1:5000, Sigma) detected with HRP-conjugated goat anti-rabbit secondary antibody (1:5000, Jackson ImmunoResearch Laboratories, West Grove, PA).

**Purification of Recombinant Proteins**—Bacillus anthracis protective antigen (PA) and fusions of RID\textsubscript{vc} to the N terminus of lethal factor (LF\textsubscript{vc}) were expressed in E. coli BL21 DE3 and purified using HisTrap and size exclusion chromatography as described previously (18). Protein concentrations were determined using the NanoDrop Spectrophotometer ND1000.

**Cell Intoxications**—HeLa cells were grown in 10-cm culture dishes to 60–80% confluence and then treated for 4 h at 37 °C, 5% CO\textsubscript{2}, with either 28 nM PA combined with 12 nM LF\textsubscript{vc} fusion proteins or with V. cholerae (multiplicity of infection = 20) as described previously (18). For microscopy, cells were seeded...
TABLE 1

Bacterial strains and plasmid used in study

| Plasmids | Relevant genotype or description | Source |
|----------|---------------------------------|--------|
| pEGFP-N3 | P<sub>CAT</sub>-egfp cloning vector for gene expression in mammalian cells, Km<sup>'</sup> | Clontech |
| pKS115  | pEGFP-N3 expressing RtxA aa 2552–3099 (RID<sub>EGFP</sub>), Km<sup>'</sup> | (18) |
| pSA4    | pKS113 - H2782A mutant, Km<sup>'</sup> | This study |
| pSA157  | pKS113 - L2851A mutant, Km<sup>'</sup> | This study |
| pSA158  | pKS113 - D2851A mutant, Km<sup>'</sup> | This study |
| pSA9    | pKS113 - C3022A mutant, Km<sup>'</sup> | This study |
| pSA64   | pKS113 - Y2807A mutant, Km<sup>'</sup> | This study |
| pSA65   | pKS113 - Y2807F mutant, Km<sup>'</sup> | This study |
| pSA9    | pKS113 - Y3015A mutant, Km<sup>'</sup> | This study |
| pSA86   | pKS113 - Y3015F mutant, Km<sup>'</sup> | This study |
| pSA87   | pKS113 - Y2807A/Y3015A double mutant, Km<sup>'</sup> | This study |
| pSA88   | pKS113 - Y2807F/Y3015F double mutant, Km<sup>'</sup> | This study |
| pABII   | F<sub>−</sub>/rpsL recombinant protein expression vector, (L<sub>Fn</sub>), Ap<sup>'</sup> | (36) |
| pKS119  | pABI expressing RtxA aa 2552–3099 (RID<sub>R</sub>), Ap<sup>'</sup> | (18) |
| pSA15   | pKS119 - H2782A mutant, Ap<sup>'</sup> | This study |
| pSA164  | pKS119 - L2851A mutant, Ap<sup>'</sup> | This study |
| pSA165  | pKS119 - D2851A mutant, Ap<sup>'</sup> | This study |
| pSA18   | pKS119 - C3022A mutant, Ap<sup>'</sup> | This study |
| pSA16   | pKS119 - Y2807A mutant, Ap<sup>'</sup> | This study |
| pSA20   | pKS119 - Y2807F mutant, Ap<sup>'</sup> | This study |
| pSA17   | pKS119 - Y3015A mutant, Ap<sup>'</sup> | This study |
| pSA21   | pKS119 - Y3015F mutant, Ap<sup>'</sup> | This study |
| pSA22   | pKS119 - Y2807F/Y3015F double mutant, Ap<sup>'</sup> | This study |
| pET15b-PA | F<sub>−</sub>/pag recombinant protein expression vector, (Protective Antigen), Ap<sup>'</sup> | (36) |
| pWM91   | ori66k orf<sub>pa</sub>, bla sacB, suicide vector, Ap<sup>'</sup> | (37) |
| pSA129  | pWM91 - RID<sub>v</sub>, aa 2552–3099, H2782A mutant, Ap<sup>'</sup> | This study |
| pBLL21  | pWM91 - RID<sub>Δ</sub>, aa 2552–3099, C3022A mutant, Ap<sup>'</sup> | This study |
| pSA41   | pΔMD-RID-EGFP, aa 2721–2954 deleted | This study |
| pSA42   | pΔCTD-RID-EGFP, aa 2943–3085 deleted | This study |

V. cholerae

| Designation | Relevant genotype or description | Source |
|-------------|---------------------------------|--------|
| KVF119      | N16961ΔnpaΔAΔhsaA, Sm<sup>'</sup> | (15) |
| KVF92       | N16961ΔnpaΔAΔhsaAΔα, Sm<sup>'</sup> | (15) |
| CCC5        | N16961ΔnpaΔAΔhsaAΔαΔACD, Sm<sup>'</sup> | (15) |
| SAHV1       | KVF119, RID-H2782A, Sm<sup>'</sup> | This study |
| SAHV2       | CCC5, RID-H2782A, Sm<sup>'</sup> | This study |
| BVG1        | CCC5, RID-C3022A, Sm<sup>'</sup> | This study |

Escherichia coli

| Designation | Relevant genotype or description | Source |
|-------------|---------------------------------|--------|
| BL21 ADE3   | F-ompT gal dcm lon hsdSB (rb- mB- A) (DE3)[lacUV5-T7 gene 1 indd3; sac] | Invitrogen |
| TOP10       | F- mcrA Δ(ntr-hsd)RMS-mcrBC (q80lacZA4 lacZA14 mupG recA araD139 Δ(aara-leu)7697 galE15 galK16 rpsL3(Sm<sup>'</sup>) endA1 λ) | Invitrogen |
| DH50 Apir   | supE4 ΔlacU169 (q80 lacZAΔ14) hsdA17 recA1 endA1 gyrA96 thi-1 relA1 lprB | (38) |
| SM10 Apir   | thi-1 leu leu I tonA lacY upc recA CRP4–2-Tc-Mu Kan<sup>'</sup> Apir | (39) |

*<sup>a</sup>, antibiotic resistance: Ap, ampicillin; Km, kanamycin; Sm, streptomycin

onto coverslips before intoxication and processed for confocal microscopy as described above.

G-LISA<sup>TM</sup> Assays—Cells treated with purified LF<sub>N</sub> fusion proteins, or bacteria were scraped off into culture medium, pelleted, washed with phosphate-buffered saline (PBS), and lysed in G-LISA<sup>TM</sup> cell lysis buffer with protease inhibitor mixture (Cytoskeleton, Denver, CO). Cell lysates were then clarified by centrifugation at 10,000 × g for 1 min. Lysates from cells treated with purified LF<sub>N</sub> fusion proteins were equalized based on total protein concentration and V. cholerae-treated cells equalized by volume. Lysates were assayed using RhoA G-LISA<sup>TM</sup> activation assay (Cytoskeleton) with data recorded at 490 nm with a Molecular Devices SpectraMax M5 microplate reader or by Western blotting to detect RhoA in lysates using mouse monoclonal anti-RhoA antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-mouse HRP-coupled antibody (1:5000, Jackson ImmunoResearch Laboratories).

Actin Cross-linking Assay—HeLa cells seeded in 6-well plates were infected with mid-log V. cholerae (multiplicity of infection = 20) in DMEM with 10% FBS at 37 °C, 5% CO<sub>2</sub>. After the addition of bacteria, plates were centrifuged at 500 × g for 5 min at room temperature, and cells were incubated for 30, 60, and 90 min at 37 °C, 5% CO<sub>2</sub>. Cells were harvested by scraping and centrifuging for 3 min at 2500 × g at room temperature. Cell pellets were washed with PBS and then boiled in 2× SDS sample buffer for 5 min. Samples were analyzed by Western blotting using anti-actin monoclonal antibody (1:1000, clone AC40, Sigma) or anti-α-tubulin (1:5000, Sigma) and anti-mouse HRP-coupled antibody (1:5000, Jackson ImmunoResearch Laboratories).

Circular Dichroism (CD)—Proteins were analyzed at 0.5 μM in 10 mM KH<sub>2</sub>PO<sub>4</sub>, 50 mM Na<sub>2</sub>SO<sub>4</sub>, pH 7.5, at 23 °C in 0.1-cm quartz cuvettes. Spectra were recorded from 190 to 250 nm using a Jasco J-815 CD spectrophotometer and Spectra Manager software. Two independent samples were prepared for each protein, and for each sample, five individual spectra were recorded in a single experiment. Spectra were then accumulated and averaged for each experiment.

Computational and Statistical Analyses—The alignment was generated using the ClustalW algorithm in the MacVector 12.0.2 software package (Cary, NC). Statistical analysis was performed using Prism 4.0c for Macintosh (GraphPad, La Jolla, CA). Processing of images from confocal microscopy was performed using Zeiss LSM Image Browser V4.2 software and CorelDraw Graphics Suite 15.0 for Windows. Histograms of
membrane localization in images were generated using ImageJ 1.43u software available at rsbweb.nih.gov (23).

RESULTS

Arg Residues Are Not Essential to RIDVc Function—Considering that RIDVc is highly efficient in inactivating RhoGTPases, it was considered most likely that this effector functions as a GTase-activating protein (GAP) acting broadly against Rho, Rac, and Cdc42 (18). In vitro GAP assays had previously demonstrated that RIDv did not have Rho-GAP activity (18). However, it remained possible that in vitro conditions did not enable RIDv to be active, particularly if the effector domain is activated in vivo, and thus further investigation using in vivo expressed RIDv was performed.

The active sites in GAPs are formed by Arg residues that protrude as finger-like structures to reach into the GTP binding pocket of a GTase. This Arg finger then lowers the activation energy barrier for the cleavage reaction of GTP by the GTase leading to GTP hydrolysis and the inactivation of the GTase (24). To determine if RIDv has an Arg finger, site-directed mutagenesis was used to change 13 Arg codons in the AD region of the RID domains revealed that AD residues 2721–3066 represent regions of significant sequence conservation (>30% identity among all sequences) as does a short polypeptide sequence from 2852–3099 fused to EGFP (18). RIDv activity was assessed by confocal microscopy of fixed cells after staining with phalloidin and DAPI. None of the 13 Arg to Ala substitutions resulted in loss of actin depolymerization upon transient transfection of HeLa cells (Table 2). Our results together with previous data from in vitro GAP assays (18) demonstrate that RIDv does not function as a GAP dependent upon an Arg finger.

Amino Acid Alignment Reveals Two Highly Conserved Regions in the AD—To focus further mutational analysis on conserved regions to identify residues essential for function, a bioinformatics approach was used to identify highly conserved regions within the AD. Previously, nine bacterial species have been shown to have MARTX toxins that carry RID domains (18). By contrast, transfection of the pEGFP-N3 vector or plasmids expressing H2782A and C3022A mutant RIDv proteins retained normal shape; whereas acidic residues (Glu and Asp) can serve as proton donors during catalysis steps (Lys and His) to resolve reaction intermediates, whereas basic residues (Lys and His) can serve as proton acceptors to either activate a substrate bond or an active site residue of an enzyme. Polar residues such as Cys, Ser, or Thr can be activated to mediate nucleophilic attacks on substrate bonds. Ser and Thr can also serve as sites of protein activation through post-translational modifications.

In this group a total of 33 codons (4 His, 5 Lys, 7 Glu, 5 Asp, 1 Cys, 9 Ser, and 2 Thr) were changed to Ala on RIDv-EGFP (pKS113) and tested for induction of cell rounding after transient transfection. Transient expression of the wild-type pKS113 plasmid and 30 of the RIDv-EGFP mutant proteins resulted in rounded EGFP-positive cells (Table 2), all of which appeared severely shrunken and rounded with poor staining of actin with phalloidin when viewed by confocal microscopy. By contrast, transfection of the pEGFP-N3 vector or plasmids expressing H2782A and C3022A mutant RIDv proteins showed polymerized actin in HeLa cells (Fig. 3A). Quantification confirmed that the loss of RIDv-dependent cell rounding due to these mutations was statistically significant and reproducible (Fig. 3B). Further mutagenesis revealed that exchanging...
Cys-3022 to Ser did not restore the cell rounding activity of RIDVc, indicating that an oxygen atom as a weak nucleophile was not sufficient to replace the strong nucleophilic sulfur atom in Cys (Table 2). The fact that the two identified RIDVc mutants, H2782A and C3022A, localize to the plasma membrane (Fig. 3A, GFP localization histograms), shows that they behave like wild-type RIDVc in terms of function of the 4HBM. Furthermore, Western blot analysis using an antibody against EGFP shows that the proteins are expressed at full-length size, although some degradation is seen with the mutants (Fig. 3C). Protein detected with anti-EGFP antibody was at levels at least comparable with wild type, although overall expression levels vary based on transfection efficiency. Thus, the defect in actin depolymerization by these two mutants is not due to a lack of expression of the EGFP fusion protein or instability.

**FIGURE 1.** Alignment of RID effector domains from various bacterial species show high similarity in N- and C-terminal regions. A, shown is a diagram of the RIDVc effector from the CPD cleavage sites 2434 to 3085 in MARTXVc. Location of the 4HBM membrane localization domain is shown. Numbering is based on annotation of the rtxA gene as determined by Lin et al. (35). Shaded regions represent regions of the AD with >30% identity across the 10 aligned RID proteins. B, shown is ClustalW alignment of the AD of 10 known or predicted RID effectors. Gray shading indicates 70% identical residues, whereas boxed residues denote the six residues identified in this study as defective for RhoA inactivation and cell rounding when changed to Ala. Asterisks label residues that were targeted for alanine mutagenesis but did not cause a defect in cell rounding. AD sequences in alignment are ordered top to bottom by percent similarity to RIDVc and are based on deduced protein sequences from publicly available genome sequences at GenBank or UniprotKB of *V. cholerae* (Vch, AAD21057.1); *V. anguillarum* (Vang, B1NY97); *Vibrio caribbenthicus* (Vcar, E3BQK8); *V. nigripulchritudo* (Vnig, F0V1C5); *P. mirabilis* (Pm1 and Pm2, B4FOY5); *Photorhabdus luminescens* subspp. *laumondii* (Plum, 2801318); *Xenorhabdus bovienii* (Xbov, D3UX88), and *Xenorhabdus nematophila* (Xnem, D3VAK8). Large gaps in sequence for Pm1 and Vnig were removed to improve alignment where indicated.

**FIGURE 2.** The activity domain of RIDVc is the smallest functional unit to induce cell rounding. HeLa cells were transiently transfected with pEGFP-N3 (A), pRIDVc-EGFP wild-type (pKS13) (B), pMD-RIDVc-EGFP (pSA441) (C), and pACTD-RIDVc-EGFP (pSA42) (D) fusion proteins as indicated. E, shown is Western blotting (WB) analysis of EGFP fusion-protein expression from transfected HeLa cells. Detection of actin was used as the loading control.
Asp-2854 Is Partially Defective for Cell Rounding during Transfection—Also in the group of putative enzymatic residues, transient expression of the D2854A mutant of RIDVc-EGFP resulted in an intermediate phenotype in which most EGFP-positive cells appeared smaller than vector controls, but 40% showed the severe rounding obvious in the wild-type-transfected cells (Fig. 4). Thus, Asp-2854 is a candidate to function with His-2782 and Cys-3022 forming a classic His-Asp-Cys catalytic triad.

Leu-2854 Is Also Partially Defective for Cell Rounding—Leu residues often mediate protein-protein interactions. In RIDVc, Leu residues could be involved in facilitating interactions of the AD with target proteins at the host cell plasma membrane or be essential for potential oligomerization of RIDVc itself. A total of nine Leu residues in the AD were targeted by alanine mutagenesis. Transfection of eight of the modified plasmids expressing Leu to Ala codon changes resulted in cell rounding indistinguishable from wild-type (Table 2), indicating that these residues are likely not involved in essential protein-protein interactions. However, L2851A was partially defective, showing intermediate rounding and fewer rounded cells compared with cells transfected with the wild-type plasmid (Fig. 4). This residue thus may function in properly forming the active site or be involved in substrate interactions.

Tyr-2807 and Tyr-3015 Can Be Changed to Phe but Not Ala—Tyr residues can also be involved in a variety of functions. They can be catalytically active such as in nucleotide reductases where the oxygen atom forms a radical during catalysis, provides important structural support, binds cofactors through hydrophobic stacking interactions of planar aromatic rings, or constitutes sites of post-translational modifications. There are three Tyr residues in the AD of RIDVc that are conserved among all 10 RID effector domains, all of which were targeted for alanine mutagenesis.

Changing Tyr-2807 or Tyr-3015 to Ala prevented actin depolymerization when expressed in HeLa cells (Fig. 5A). By contrast, substitution of Ala for Tyr-2722 did not affect actin depolymerization (Table 2). However, when Tyr-2807 and Tyr-3015 were changed to Phe, these mutants retained cell rounding activity (Fig. 5A). Because Tyr can be a site of post-translational modifications, it was considered that modification of

FIGURE 3. RIDVc mutants H2782A and C3022A prevent cell rounding in HeLa cells. A, HeLa cells were transfected with pRIDVc-EGFP (pKS113) expressing wild-type or mutant RIDVc fusion proteins as indicated. Cells were stained with rhodamine-phalloidin and DAPI. Histograms in the right panel display green fluorescence in arbitrary units (AFU) of a cross-section (white bar, length in pixels [px]) indicating membrane localization. Insets represent the phenotype of round cells for RIDVc mutants and pEGFP-N3 control. B, shown is quantification of cell morphology based on a total of 100–300 EGFP-positive cells. Data represent the mean and S.D. of three independent transfections. C, shown is Western blotting analysis of EGFP fusion-protein expression from transfected HeLa cells. Detection of actin was used as loading control. ***, p < 0.0001.

FIGURE 4. RIDVc mutants L2851A and D2854A cause intermediate cell rounding in HeLa cells. A, HeLa cells were transfected with pRIDVc-EGFP (pKS113) expressing wild-type or mutant RIDVc-EGFP fusion proteins as indicated. Cells were stained with rhodamine-phalloidin and DAPI. Histograms in the right panel display green fluorescence in arbitrary units (AFU) of a cross-section (white bar, length in pixels [px]) indicating membrane localization. Insets represent the phenotype of round cells for RIDVc mutants and pEGFP-N3 control. B, shown is quantification of cell morphology based on a total of 100–300 EGFP-positive cells. Data represent the mean and S.D. of three independent transfections. Intermediate phenotype refers to cells that are shrunken but normal in shape compared with normally sized and shaped pEGFP-N3-transfected cells. C, shown is Western blotting (WB) analysis of EGFP fusion-protein expression from transfected HeLa cells. Detection of actin was used as loading control. ***, p < 0.001; **, p < 0.01; *, p < 0.05.
either Tyr might partially activate RID\textsubscript{VC} in a manner sufficient to induce actin depolymerization. However, a double mutation in which both Tyr-2807 and Tyr-3015 were changed to Phe also did not prevent actin depolymerization (Fig. 5A). Western blot analysis shows that the fusion proteins are stably expressed in HeLa cells and that Tyr to Phe mutants display expression levels slightly lower than wild-type, whereas the Tyr to Ala mutants are expressed at higher levels compared with wild-type (Fig. 5B). Altogether, these results likely indicate that these Tyr residues may be necessary for hydrophobic packing of the protein or they may be involved in interactions with potential cofactors and binding partners by stacking of the planar hydrophobic face.

Mutation of Important Catalytic Residues Prevents RhoA Inactivation but Does Not Alter the Overall Structure of RID\textsubscript{VC}—Rapid screening of site-directed mutants by transient transfection identified six residues that contribute to RID\textsubscript{VC} function. To link these residues to RhoA inactivation, recombinant proteins bearing the Ala or Phe substitutions were purified fused to the first 254 aa of \textit{B. anthracis} lethal factor for delivery to cells by PA (Fig. 6A). This LF\textsubscript{N}-PA protein bioporter system developed for delivery of heterologous antigens (27) has been successfully adapted to deliver functional RID\textsubscript{VC} to epithelial cells in the absence of the remainder of the large MARTX\textsubscript{VC} toxin. After delivery by PA, LF\textsubscript{N}RID\textsubscript{VC} induces both inactivation of RhoA and actin depolymerization (18), and its function is dependent upon the presence of the 4HBM (20).

Because changing aa in a protein can potentially alter its structure, the influence of the mutations on the structural composition of LF\textsubscript{N}RID\textsubscript{VC} was assessed using CD spectroscopy. CD absorbance spectra of LF\textsubscript{N}RID\textsubscript{VC} wild-type and mutant proteins were recorded between 190 and 250 nm. There was no significant structural difference between wild-type and the fully inactive H2782A and C3022A (Fig. 6B), the intermediate active L2851A and D2854A mutants (Fig. 6C), or the Tyr mutant RID\textsubscript{VC} proteins (Fig. 6D).

Next, HeLa cells were incubated with purified LF\textsubscript{N}RID\textsubscript{VC} fusion proteins, controls, and PA for 4 h at 37 °C. As expected, HeLa cells treated with wild-type LF\textsubscript{N}RID\textsubscript{VC} rounded only when incubated in the presence of PA (Fig. 7A). By contrast, all six Ala mutant LF\textsubscript{N}RID\textsubscript{VC} proteins did not cause actin depolymerization, and cells appeared phenotypically normal in shape (Fig. 7A), whereas cells treated with Y2807F and Y3015F rounded (Fig. 7A). That the L2851A and D2854A mutant proteins did not show partial cell rounding as seen during transient transfection indicates that these have partially activity only upon overexpression.

Commercially available RhoA G-LISA\textsuperscript{TM} assays were then used to quantify the active GTP-bound form of RhoA (RhoA-GTP) from intoxicated cells. In wild-type LF\textsubscript{N}RID\textsubscript{VC}-treated HeLa cells, RhoA-GTP levels were reduced by 55–65% compared with RhoA-GTP levels in LF\textsubscript{N} control-treated cells (Fig. 7, B–D). In HeLa cells treated with LF\textsubscript{N}RID\textsubscript{VC} bearing Ala mutations in His-2782, Cys-3022, Leu-2851, or Asp-2854, RhoA-GTP levels were not significantly different from LF\textsubscript{N} control-treated cells demonstrating that these four RID\textsubscript{VC} mutants are defective in Rho inactivation activity (Fig. 7, B and C). The Y2807A and Y3015A mutants also did not inactivate RhoA, whereas the Y2807F and Y3015F LF\textsubscript{N}RID\textsubscript{VC} mutant-treated cells had levels of RhoA-GTP comparable with wild-type LF\textsubscript{N}RID\textsubscript{VC}-treated cells (Fig. 7D). Cells treated with the YYFF double mutant were also significantly reduced in RhoA-GTP levels, but levels were also significantly higher than wild-type LF\textsubscript{N}RID\textsubscript{VC}-treated cells.

**FIGURE 5.** YA but not YF RID\textsubscript{VC} mutants prevent cell rounding. A, HeLa cells expressing pRID\textsubscript{VC}-EGFP (pKS113) wild-type or Tyr-mutant RID\textsubscript{VC}-EGFP fusion proteins as indicated were stained with rhodamine-phalloidin and DAPI. Insets represent round cells observed in transfections with YA RID\textsubscript{VC}-EGFP mutants or pEGFP-N3 control. B, Western blotting (WB) analysis of EGFP fusion-protein expression from transfected HeLa cells is shown. Detection of actin was used as loading control.
indicating an intermediate defect in Rho inactivation activity (Fig. 7D), resulting in cells that may be slightly less rounded (Fig. 7A).

For all assays, Western blotting of cell lysates generated for the RhoA G-LISA™ experiments with anti-RhoA antibody confirmed that there was no change in the overall amount of RhoA or a shift in its electrophoretic mobility consistent with previous findings that RIDVC does not change expression or cause a covalent modification of RhoA (Fig. 7, B–D, and Ref. 18). Altogether, we conclude that mutation of His-2782 Asp-2854, and Cys-3022 significantly prevented RhoA inactivation in cells treated with RIDVC and that the effects of these mutants are due to being catalytically deactivated enzymes rather than disturbed in their global structural integrity. The significant reduction of RIDVC activity observed for the Tyr to Ala mutants as well as for the L2851A mutant are not due to overall structural changes, and thus these residues may be essential to form a binding surface or help properly orient the active site residues but may not be involved in the catalytic activity of RIDVC.

The H2782A and C3022A Mutants Also Prevent Cell Rounding and RhoA Inactivation in the Context of the Holotoxin Delivered by V. cholerae—Rho inactivation by RIDVC normally occurs during bacterial infection after release to the cytoplasm by autoprocessing from the translocated MARTXVC holotoxin. To assess the consequences of a deactivated RIDVC in the context of the natural delivery system, the H2782A and C3022A mutations that were fully defective for activity during transient transfection and direct cytosolic delivery by the PA-LFN system were transferred onto the V. cholerae chromosome using double homologous recombination. As a first assessment, the RIDVC mutations were transferred onto the chromosome of V. cholerae CCO5, a strain modified to have an in-frame deletion in the rtxA gene to remove sequences for the ACDVC, resulting in loss of actin cross-linking activity (15). When co-incubated with cells at an multiplicity of infection = 20 for 4 h, the rtxA null strain KFV92 (15) reduced overall RhoA activation and levels of total RhoA compared with mock-inoculated controls, indicating a general cellular response to the bacteria independent of the toxin (Fig. 8B). By contrast, control strain CCO5 induced toxin-dependent actin

FIGURE 6. Point mutants do not alter the overall structure of RIDVC. A, shown is SDS-PAGE of 2 μg of purified recombinant LFN RIDVC wild-type or mutant proteins. B–D, shown are CD spectra of 0.5 μM LFN fusion proteins. The spectra shown were averaged from five separate readings for two independent samples.

FIGURE 7. RIDVC alanine point mutants prevent inactivation of RhoA. HeLa cells treated with 28 nM PA plus 12 nM of the indicated LFN fusion proteins for 4 h were stained with rhodamine-phalloidin and DAPI and visualized by confocal microscopy (A) or lysed and assayed for RhoA-GTP (A490) using RhoA G-LISA™ assays (B–D). Western blot detection of total recovered RhoA from cell lysates is shown below the bar diagrams. ***, p ≤ 0.0001; **, p ≤ 0.001; *, p ≤ 0.01; n.s., not significant.
Modification of CCO5 to incorporate the H2782A and C3022A mutations generated strains SAHV2 (CCO5-H2782A) and BGV1 (CCO5-C3022A), respectively. Consistent with data from transient transfection and direct cytosolic delivery of LF_{Vc} RID_{Vc}, the two mutations H2782A and C3022A in the MARTX_{Vc} holotoxin prevented toxin-induced cell rounding (Fig. 8A), suggesting that both mutants are catalytically deactivated under natural infection conditions. Notably, although BGV1 was also defective for RhoA inactivation, BGV1 activated under natural infection conditions. Notably, although MARTX_{Vc} toxin function suggest that the RID_{Vc} and ACD_{Vc} effectors act in synchrony for rapid actin depolymerization with RID_{Vc}, eliminating activated RhoA, resulting in generation of an increased concentration of monomeric G-actin that then serves as the substrate for ACD_{Vc}-dependent actin cross-linking (28).

To assess the effect of RID_{Vc} inactivation on ACD_{Vc} activity, the H2782A mutation was transferred onto the chromosome of _V. cholerae_ strain KFV119 in which the ACD_{Vc} remains intact generating strain SAHV1. The H2782A mutation was chosen because studies described above showed this protein has no residual RhoA inactivation activity. HeLa cells were co-incubated with bacterial strains, and cell lysates were analyzed by Western blotting using monoclonal actin antibody to detect the mobility shift in actin as it is cross-linked. Over a time course of 90 min, there was no difference in the detectable amount, dynamics, or patterns of actin cross-linking between _V. cholerae_ strain KFV119 that has a functional RID_{Vc} compared with strain SAHV1 with a catalytically inactive RID_{Vc} effector domain (Fig. 9). We, therefore, conclude that, in contrast to prevailing models, the rate of cross-linking by ACD_{Vc} in vivo is not affected by RID_{Vc} and that the two domains function independently in their ability to compromise the actin cytoskeleton.

**DISCUSSION**

In this study we undertook a site-directed mutagenesis approach to identify residues of the _V. cholerae_ MARTX toxin essential for its activity in inactivating RhoA. Our sequence analysis revealed a total of 10 members of the RID effector domains. Among the nine MARTX toxins that carry a RID (or multiple domains in the case of _P. mirabilis_), only the RID effector domain from MARTX_{Vc} has been experimentally demon-
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strated to cause disassembly of the actin cytoskeleton and small RhoGTPase inactivation (18). Genetic evidence analyzing rtxA null mutants from *Vibrio vulnificus*, *Vibrio anguillarum*, and *V. nigripulchritudo* have linked the MARTX toxins with actin depolymerization and cell rounding, indicating that these toxins may also inactivate RhoGTPases through the activity of their RID effectors (25, 29, 30). Global assessment of the alignments reveals that these proteins are strongly conserved within the 4HBM region (up to 89% identity across ∼85 residues (20)) including strong conservation of the Lys-2577 and Arg-2630 residues required for plasma membrane localization (19).

As previous studies have demonstrated that the 4HBM region is sufficient for membrane localization and the AD domain is sufficient for cell rounding (20), this study focused on identification of residues within the AD subdomain required for RhoGTPase inactivation and actin depolymerization. Sequence alignment shows that the AD subdomain is likely structurally split into three units that are highly conserved connected by less conserved regions that differ not only in sequence but also in size. Thus, although it is suggested that our studies here will apply to all RID proteins regarding identification of catalytic residues, it is possible that nonconserved regions may confer altered target specificity to the RID effector domains from other species, and thus it may not necessarily be true that all RID proteins will have conserved function during infection.

The critical residues identified, His-2782, Leu-2851, Asp-2854, and Cys-3022, are each found in the highly conserved portions of the protein, indicating that the three conserved structural units of the protein are brought together in the final structure to create a single active site. Such a structural configuration and the requirement for the His and Cys has been previously predicted to be important for catalysis through an *in silico* analysis (21). The earlier bioinformatics study further linked the RID\textsubscript{Vc} with several type III secretion effectors, notably IcsB from *Shigella flexneri* and BopA from *Burkholderia pseudomallei*. Both proteins have been associated with the ability of intracellular bacteria to evade autophagy, although the catalytic activity leading to this evasion is unknown (31, 32).

The shared feature of these enzymes is that they all display homology to circularly permuted papain-like-fold proteins of the PPPDE (permuted papain fold peptidases of dsRNA viruses and guakryotes) family in which the catalytic Cys is located close to the C terminus of the protein (21). This family is also known as Clan CE of cysteine endopeptidases in the MEROPS database (33). Enzymes in this clan perform hydrolytic reactions as cysteine proteases or alycraferases and may play a potential role in ubiquitin signaling pathways (34). Our identification of His-2782 and Cys-3022 as essential for RID\textsubscript{Vc} activity is consistent with RID\textsubscript{Vc} placement in this Clan.

It is notable that all members of the Clan CE peptidases share an active site structure composed of His-(X15–19)-Asx/Glx and Gln-(X5–6)-Cys. In these proteases, the Asx/Glx functions to properly orient the imidazole ring of the His to the catalytic site (33). Previous bioinformatics analyses of RID\textsubscript{Vc} failed to identify a residue that would orient the His, and the study concluded that RID\textsubscript{Vc} IcsB, BopA, and related proteins are not typical circular permuted thiol proteases in that they have only a His-Cys catalytic dyad (21). Using a revised alignment strategy that was manually inspected to adjust for alignment gaps of RID proteins of differing sizes, we identified a small 17-aa polypeptide sequence that was conserved in all RID proteins and included 2 Asp and 2 Gln residues as potential residues to function as part of a catalytic triad. Alanine mutagenesis of conserved residues in this short aa region revealed that Asp-2854 was significantly defective for RID\textsubscript{Vc} activities, consistent with a function to orient His-2782 in an active site. This residue is located 72 residues downstream from His-2782 in RID\textsubscript{Vc}, but the distance ranges from 40 to 73 aa in RID proteins among other MARTX toxins (Fig. 1B). Thus, the peptide region containing Asp-2854 must fold toward the active site with unique subdomains formed by the intervening residues in the different sized RID proteins. Tyr-2807 and Leu-2851 may participate in the final orientation of the catalytic residues or contact of the substrate as they contribute to RID\textsubscript{Vc} activity without affecting tertiary structure.

In Clan CE enzymes the active sites have a Gln 5–6 residues in front of the catalytic Cys that functions as the oxyanion hole to stabilize the main-chain carbonyl group of the substrate protein by providing polar interactions (33). The Tyr residue seven residues ahead of Cys-3022 was proposed to serve this function for RID\textsubscript{Vc} (21). Indeed, Tyr-3015 in RID\textsubscript{Vc} could be changed to Phe but not to Ala, consistent with a role in providing polar interactions necessary for an oxyanion hole. However, it is notable that in all Clan CE peptidases, the oxyanion hole is formed by a Gln residue, not Tyr. Adjacent to Tyr-3015 in RID\textsubscript{Vc} is Gln-3016, placing it in the proper position 6 residues in front of Cys-3022. It is possible that the proper function of Tyr-3015 is in fact to orient Gln-3016 to the active site, where it can stabilize the substrate. This residue, however, was not identified by either a bioinformatics study or our analysis of 100% conserved residues because it is found as a His in some RID proteins and as a His, Lys, or Asn in other bacterial proteins associated with this family by bioinformatics.

Overall, these studies suggest that RID\textsubscript{Vc} is the representative member of a new subfamily of the Clan CE circular permuted peptidases, all of which have an Asp residue to orient the His located in a distant subdomain that is then oriented back toward the His and an oxyanion hole formed in part by a Tyr adjacent to Gln or His spaced seven residues ahead of the catalytic Cys. Future studies using crystallography will be required to determine if these indeed form a new family and if the identified residues associated into a coordinated active site.

The identification of critical residues in the RID\textsubscript{Vc} facilitated examination of the function of RID\textsubscript{Vc} in the context of the holotoxin without concern for secondary effects on autoprocessing or toxin delivery that could complicate the analysis. It was found that the H2782A mutant was completely defective for toxicity against cells, resulting in no obvious effects on cytoskeleton assembly or cell shape, at least within the first 4 h. These data confirm that H2782A is essential for RID\textsubscript{Vc} function and also demonstrates that other domains of the MARTX\textsubscript{Vc} in particular the αβ-hydrolase domain and CPD, do not cause overt toxicity in the first 4 h after bacterial exposure. By contrast, the C3022A mutant showed an intermediate effect on RhoA inactivation in the context of the holotoxin and a slight change in...
actin condensation at the periphery of some cells, suggesting this mutation may bind the target in vivo when properly delivered to cells without either EGFP or LFα tags. However, the H2782A mutation clearly does not have this residual binding activity, suggesting H2782A may affect substrate binding in addition to catalysis.

A key question that has arisen regarding MARTX toxins is why these toxins have such distinct arrays of toxin effectors depending on strain or species and whether unique combinations of toxin effectors are synergistic. Data using the deactivated RIDVc in the MARTXVc holotoxin shows that there is no enhanced effect on actin cross-linking by the RIDVc. These data complement previous observations that the level of RhoA-GTP is not affected by the presence or absence of an ACDV (18). Thus, these appear to be fully independent activities that contribute to actin depolymerization by different strategies. A possibility remains that RhOGTase inactivation may induce other downstream signaling changes in cells that could affect aspects of cell biology in addition to promoting actin depolymerization.

In total, the work presented here clearly demonstrates the importance of a His-Asp-Cys catalytic triad in RIDVc as being essential for cell rounding and RhoA inactivation. Furthermore, it was shown that functional RIDVc is not required for efficient actin cross-linking, demonstrating that the two cell rounding activities of MARTXVc are fully redundant. In the future, the exact biochemical mechanism responsible for these activities needs to be characterized and the target of RIDVc identified.

Acknowledgments—We thank Kevin Ziolo and Jayme Kwak for technical assistance. DNA sequencing was carried out by the Northwestern University Genomics Core Facility, imaging work was performed in the Northwestern University Cell Imaging Facility, and circular dichroism measurements were carried out in the Keck Biophysics Facility at Northwestern University.

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