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MINIREVIEW

MARTX, Multifunctional Autoprocessing Repeats-in-Toxin Toxins[▽]

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The repeats-in-toxin (RTX) exoproteins are a diverse collection of proteins exported via type I secretion by gram-negative bacteria. These proteins include the pore-forming RTX toxins typified by α-hemolysin of *Escherichia coli* and *Bordetella pertussis* adenylate cyclase toxin (ACT) and also secreted enzymes such as metalloproteases and lipases, S-layer proteins, a nodulation signaling factor, and the Fe-regulated FrpC "clip-and-link" toxin of *Neisseria meningitidis* (24, 27).

In 1999, a new toxin from *Vibrio cholerae* was discovered that was initially categorized in the RTX toxin family and simply named "the RTX toxin" or VcRtxA (22). This toxin is produced by nearly all clinical and environmental isolates of *V. cholerae*, including the El Tor O1 strains responsible for the current cholera pandemic plus a broad selection of O139 and non-O1/non-O139 strains (7, 9, 10, 13). The toxin has been associated with increased epithelial cell damage in a mouse lung infection model (14) and has also been associated with increased virulence in a mouse gut infection model, although its role in intestinal disease is currently unclear (26). Recently, a related toxin from *Vibrio vulnificus* has been described and found to be important for virulence in mouse models (21, 23).

On-going study of the mechanism of action of the *V. cholerae* toxin in vitro has revealed that it is a totally novel type of toxin with many features that distinguish it from other RTX toxins. In addition, release of genomic sequences from human, marine, and insect pathogens has indicated that the toxins from *V. cholerae* and *V. vulnificus* are not unique but rather are the first characterized members of a new family of the RTX exoproteins. Within this new family, the structural properties of the toxins are highly conserved, but the catalytic activities carried are predicted to vary since the toxins are assembled as mosaics of 10 activity domains assorted among the various toxins.

In this review, the structure and function of the *V. cholerae* toxin are discussed in detail, revealing that this is a novel toxin distinct from the other RTX toxins. The structural features that define the new family and the mosaic structure of the activity domains are also discussed. Overall, it is shown that the *V. cholerae* toxin is a *m*ultifunctional *a*utoprocessing RTX toxin, renamed MARTX$_{Vc}$, that represents a larger group of MARTX toxins produced by at least eight gram-negative species.

NOVEL REPEAT STRUCTURE OF MARTX TOXINS

A common property of all RTX pore-forming toxins is their large size; their molecular masses range from 100 to 177 kDa (24). At the C terminus of these large proteins are "GD-rich" nonapeptide repeats (GGXGXDX[L/I/V/W/Y/F]X, where X is any amino acid), which are the defining characteristic of the RTX exoproteins (20). These repeats have been shown to fold to form a stable β-roll structure that binds Ca^{2+} (3) and are thought to be involved in proper insertion of the toxins into the eukaryotic cytoplasmic membrane (29).

Sequences of novel A, B, and C repeats. The MARTX$_{Vc}$ toxin is distinguished from other RTX toxins both by its size and by its novel repeat structure. Compared to the other RTX toxins, MARTX$_{Vc}$ is much larger, containing 4,545 amino acids (aa) and having a predicted molecular mass of more than 485 kDa (22). The related toxin from *V. vulnificus* (MARTX$_{Vv}$) is also extremely large, containing 5,206 aa and having a predicted molecular mass of 556 kDa, and it is now recognized as the largest single polypeptide toxin, surpassing MARTX$_{Vc}$ (21). The large size of these proteins is due in part to the fact that about 25% of the primary sequences is composed of glycine-rich repeats, but it is notable that these repeats are distinct from the nonapeptide repeats of other RTX toxins. These repeats fall into three classes that differ in overall sequence but share a common G-7X-GXXN central motif. The first class, the "A repeats," were not identified in the original annotation of MARTX$_{Vc}$ (22) and thus are shown in Fig. 1. These are 20-residue repeats with the consensus sequence GXXG(N/D)(L/I)(T/S)FXGAG(A/G)XNX(L/I)X(RH), featuring the central motif (underlined). There are 16 copies of this repeat in two groups at the extreme N terminus between residues 73 and 700 (Fig. 2).

The second class of repeats was originally designated the novel repeats, but here these repeats are designated the "B repeats" to distinguish them from the A repeats also located within the N terminus. The original annotation reported 29 copies of the 19-aa B repeat (22); however, a realignment of the repeats identified 34 consecutive copies at aa 697 to 1357 with the consensus sequence T(K/H)VGDGX(S/T)VAVMXGXAN(I/V)X, revealing the central motif (underlined) (data not shown).

The third class is the RTX repeats present at the C terminus of the protein originally identifying MARTX$_{Vc}$ with the RTX family. However, while MARTX$_{Vc}$ does have recognizable repeats, they are separated by 9 aa and have the central core G-7X-GXXN motif, making them an 18-aa repeat with similarities to both the nonapeptide RTX repeat

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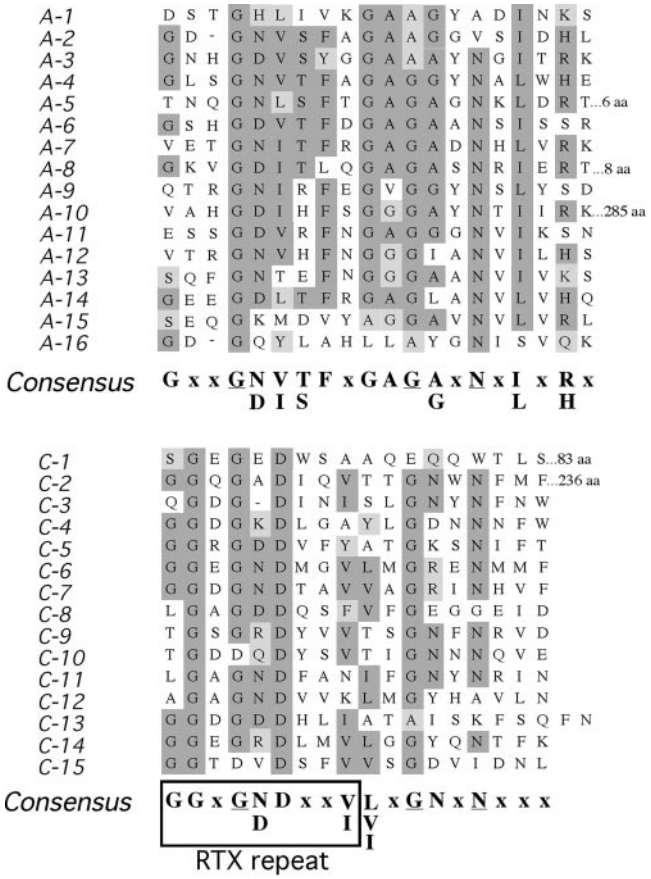

FIG. 1. A and C repeats of MARTX$_{Vc}$. Repeats were aligned using MacVector 7.0 (Oxford Molecular Group). Residues that align with the consensus are dark gray, while conserved residues are light gray. The consensus sequence is shown below the repeats, where x is any amino acid. The 9-aa RTX repeat is in the consensus sequence of the C repeat as indicated. Residues that form the core motif G-7X-GXXN are underlined in the consensus sequence.

and the A and B repeats. In the original report, 11 repeats were identified (22). After adjustment of the consensus sequence to align the core motif, 15 copies of this C-terminal repeat, designated the "C repeat" to differentiate it from the 9-aa RTX repeat, were identified; these copies included 2 copies that were independent copies, whereas the other 13 copies occur in succession (Fig. 1 and 2). Thus, MARTX$_{Vc}$ is clearly distinct from other RTX toxins as it is comprised of glycine-rich repeats that are not present in any of the other RTX exoproteins.

Repeat structure is conserved in 12 MARTX toxins. Utilizing the A, B, and C repeat sequences of MARTX$_{Vc}$ as the query sequence in a PSI-BLAST GenBank database search (1), MARTX$_{Vv}$ and 10 other putative toxins that share the repeat structure of MARTX$_{Vc}$ were identified (Fig. 2). The deduced protein products of the genes are all predicted to be large, ranging from 3,212 to 5,206 aa long. Alignment of the deduced sequences of all 12 MARTX members reveals regions of strong sequence conservation throughout the N terminus with 45 to 95% sequence identity and 62 to 97% sequence similarity. All but two toxins have the same number of A and B repeats. The exceptions are the putative toxin from *Aeromonas hydrophila* (MARTX$_{Ah}$), which is missing one A2 repeat due to an exact 18-aa gap in the alignment, and *Yersinia enterocolitica* MARTX$_{Ye}$, in which the N-terminal arm is shorter due to the loss of six A1 repeats. The region of alignment extends another 600 aa beyond the repeats and includes a putative coiled-coil region of unknown function. The C-terminal repeats are likewise highly conserved (62 to 99% sequence identity), and all members have 15 copies.

Analysis of complete genomes (when available) showed that most bacterial species identified have a single MARTX toxin; the only exception is *Photorhabdus luminescens*, which has four intact MARTX toxin genes that could produce four toxins (MARTX$_{Pl1}$ to MARTX$_{Pl4}$), three of which are nearly identical (12). Many of the identified bacterial species also have open reading frames that could encode other large RTX exoproteins, but the putative toxins do not have the same novel A, B, and C repeat structure and thus likely represent other new families of RTX toxins. Overall, the presence of the highly conserved A, B, and C repeats defines this new MARTX family of toxins.

CONSERVATION OF RTX GENE CLUSTERS

"Atypical" type I secretion. All RTX family exoproteins are exported directly from the bacterial cytosol to the extracellular milieu by type I secretion systems (T1SS). For all other families of RTX exoproteins, the T1SS consist of three components: a homodimer of an inner membrane transport ATPase, a trimer of a transmembrane linker protein, and an outer membrane porin that is either a specialized porin or the common porin TolC (24). In both *V. cholerae* and *V. vulnificus*, the *rtxA* toxin genes that encode the MARTX toxins are organized into a six-gene cluster such that *rtxA* is transcribed in a single operon downstream of *rtxC* (described below) and a hypothetical gene *rtxH* and is divergently transcribed from conserved T1SS genes (5, 21, 23). However, the linked three-gene T1SS operons include two transport ATPase genes, *rtxB* and *rtxE*, in addition to a transmembrane linker gene, *rtxD* (Fig. 3). Experiments have shown that extracellular secretion of MARTX$_{Vc}$ requires *rtxD*, the unlinked gene *tolC*, and both *rtxB* and *rtxE* (4, 5). Furthermore, both RtxB and RtxE require intact mononucleoside binding sites, indicating that hydrolysis of ATP by both proteins is necessary for MARTX$_{Vc}$ export (5). Thus, type I secretion of MARTX$_{Vc}$ is "atypical" in that it requires a heterodimeric inner membrane transport ATPase complex in addition to the transmembrane linker RtxD and outer membrane porin TolC. In *V. vulnificus*, a transposon insertion in *rtxE* has been reported to ablate MARTX$_{Vv}$-associated cytotoxicity, indicating that MARTX$_{Vv}$ secretion is also dependent upon at least RtxE (21). Similarly, most of the other MARTX toxin-encoding *rtxA* genes are organized into six-gene clusters composed of two divergent operons and would be predicted to be exported by a T1SS that includes a heterodimeric ATPase complex (5), suggesting that secretion by an atypical T1SS is a conserved feature for this family of toxins.

One exception to this tight linkage of the secretion apparatus with the *rtxA*-like genes is found in *P. luminescens*, where two functional *rtxA* copies and two pseudogene copies occur in tandem linked to the *rtx* locus, while four *rtxA*

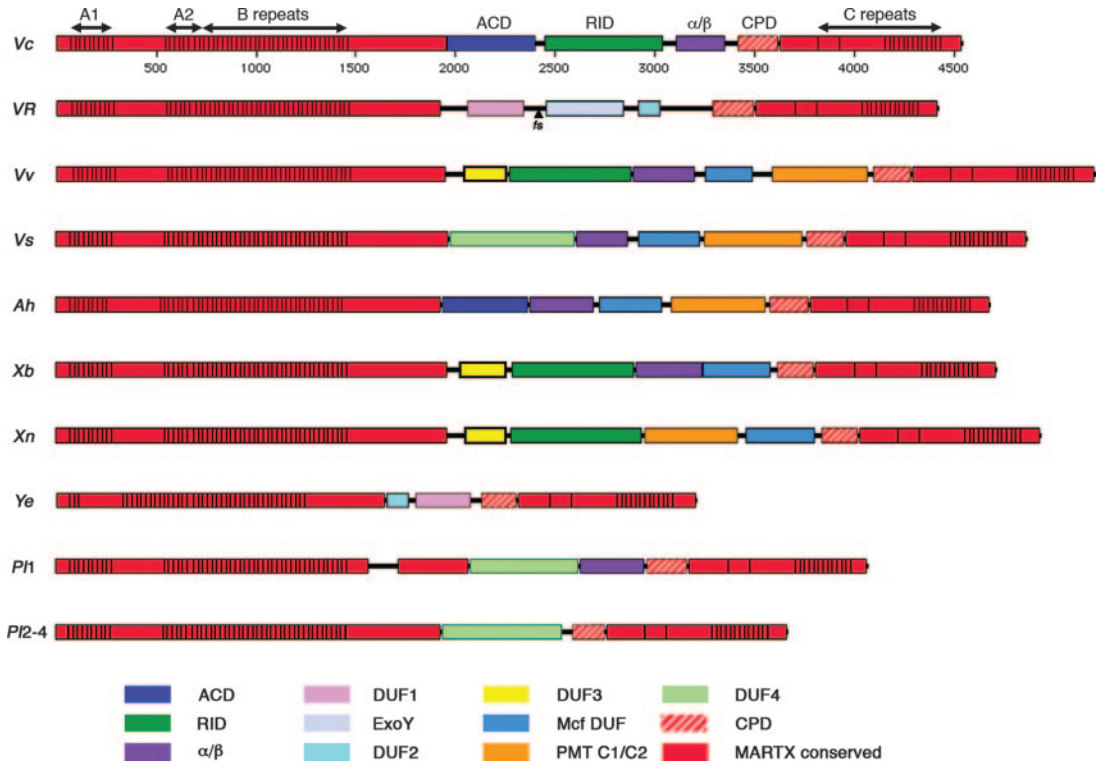

FIG. 2. Representative MARTX$_{Vc}$ toxin is similar to MARTX$_{Vv}$ and 10 other putative MARTX toxins. The red regions are highly conserved among the nine MARTX toxins shown; this includes but is not limited to the repeat regions (indicated by vertical lines) labeled at the top as A1, A2, B repeats, and C repeats as described in the text. The central activity domains for each toxin are color coded as indicated at the bottom, where abbreviations for genes with known or proposed functions as defined in text and domains with unknown functions (DUF) are also indicated. Genes were identified using PSI-BLAST (1), followed by analysis of independent protein sequences downloaded from the National Center for Biotechnology Information database, with the following accession numbers: *V. cholerae* N16961 (*Vc*), AAD21057.1 (16b); *V. cholerae* RC385 (*VR*), NP_937086.1; *V. vulnificus* YJ016 (*Vv*), NP_937086.1 (6a); *V. splendidus* 12B01 (*Vs*), ZP_00989505; *A. hydrophila* subsp. *hydrophila* ATCC 7966 (*Ah*), YP_855898 (29a); *Y. enterocolitica* type 0:3 (*Ye*), CAJ90394; and *P. luminescens* subsp. *laumondii* TT (*Pl*1 and *Pl*2-4), NP_928648, NP_928647, NP_930444, and NP_930545 (12). The sequences of *Xenorhabdus bovienii* (*Xb*) and *Xenorhabdus nematophila* ATCC 19061 (*Xn*) were downloaded from www.xenorhabdus.org (16a), and a comparative analysis was performed using BLAST2 (33a). Diagrams were drawn to scale using MacVector 7.0 (Oxford Molecular Group).

copies (two functional genes and two pseudogenes) are located at unlinked loci (12). In *Y. enterocolitica*, the three-gene operon *rtxHCA* has been identified in the unfinished type 0:3 genome, but genes for a type I export apparatus were not present in the same contiguous sequence. As this toxin gene is not found in the fully sequenced genome of strain 8081 (34), no further search for unlinked secretion genes was possible.

Posttranslational modification by acylation? Another property shared by the RTX pore-forming toxins but not by all RTX exoproteins is posttranslational modification by the addition of acyl groups to lysines (24). For both *E. coli* α-hemolysin and *B. pertussis* ACT, acylation has been shown to be essential for toxin activity against target eukaryotic cells (2, 33). The *V. cholerae* gene *rtxC* encodes a protein similar to the acyltransferase required for maturation of *E. coli* α-hemolysin and is located in the same operon as the *rtxA* toxin gene (5, 22). In *V. cholerae*, creation of an *rtxC* mutant that is nonpolar on the downstream *rtxA* gene has proven to be technically challenging, suggesting that production of nonacylated MARTX$_{Vc}$ is somehow detrimental to bacterial viability (B. K. Boardman and K. J. F. Satchell, unpublished observations). Recently, successful disruption of *V. vulnificus rtxC* was reported and found to have no effect

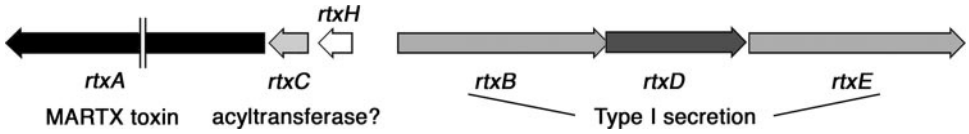

FIG. 3. Generalized organization of *rtx* gene clusters, except for *Y. enterocolitica* and *P. luminescens* as noted in the text. The operon structure as drawn is modified from reference 5, with the gene designations for the left-oriented *rtxHCA* operon based on the annotation of *Y. enterocolitica rtx* genes (NCBI, accession number CAJ90394) and the gene designations for the right-oriented *rtxBDE* operon previously proposed for *V. cholerae* and *V. vulnificus rtx* genes (modified from reference 5; 21).

on virulence despite the fact that disruption of *rtxA* decreases the 50% lethal dose of *V. vulnificus* by 2 to 5 log units (18, 21, 23). This result strongly suggests that acylation is not required for $MARTX_{Vv}$ toxicity and thus may not be essential for all MARTX toxins.

$MARTX_{Vc}$ IS A MULTIFUNCTIONAL CELL-ROUNDING TOXIN

The majority of RTX toxins are pore-forming toxins that insert into the target cell plasma membrane and form oligomeric pores, eventually leading to alteration of membrane permeability and cell lysis (33). However, a major feature that distinguishes $MARTX_{Vc}$ from the other RTX toxins is that it is not a pore-forming toxin. Indeed, standard assays for cell lysis and altered membrane permeability demonstrated that $MARTX_{Vc}$ does not cause cell lysis (16). Furthermore, neither $MARTX_{Vc}$ nor any of the other putative MARTX toxins have a conserved hydrophobic domain for generation of a pore structure like that found in other RTX toxins (22).

Instead, $MARTX_{Vc}$ causes rounding of nonpolarized cells and loss of the integrity of paracellular tight junctions of polarized cells, yet the cells remain viable (15, 16, 22). Many toxins that cause cell rounding without directly affecting cell viability target the actin cytoskeleton. Consistent with this, 2 h after exposure to *V. cholerae*, cells in culture cease to bind phalloidin, indicating that no actin stress fibers remain (16). Thus, this toxin is associated with depolymerization of actin and loss of cell structure, not pore formation. This loss of cell structure is due to two distinct activities of $MARTX_{Vc}$.

Actin cross-linking. A Western blot of lysates from cells infected with *V. cholerae* revealed that actin itself is one target of toxin action. Coincident with the disassembly of actin, the actin monomers become covalently linked into multimers, a phenotype called actin laddering (16). The domain essential for this activity has been located between aa 1963 and 2419. Deletion of this region, called the "actin cross-linking domain (ACD)," from $MARTX_{Vc}$ eliminated laddering, demonstrating that this region is essential. Furthermore, cross-linking of actin occurred in cells transiently expressing the domain separated from the remainder of the toxin, a result showing that this domain is sufficient for the actin laddering phenotype (31). Finally, purified ACD has been demonstrated to cross-link actin in vitro in the absence of other cellular proteins, demonstrating that the ACD is an enzyme that directly binds actin and introduces the covalent cross-link (8).

Rho GTPase inactivation. Since the ACD is essential for actin cross-linking, it was expected that deletion of the ACD would also inactivate cell rounding. However, elimination of the ACD did not ablate cell rounding, revealing that this large toxin has a second cell-rounding activity (31). Further investigation has shown that this second activity is mediated through inactivation of the small Rho GTPases Rho, Rac, and CDC42 (32). This activity has been mapped to a specific domain of the toxin located at aa 2552 to 3099, termed the "Rho GTPase inactivation domain (RID)." When transiently expressed in cells, the RID caused cell rounding, and purified RID induced both cell rounding and Rho inactivation when it was delivered directly to the cytosol of cells (32). The mechanism of Rho GTPase inactivation has not yet been determined, but current data suggest that the RID targets the Rho GTPase signaling pathways rather than the small GTPases directly by a mechanism that is not shared with other known Rho-inactivating toxins (32). Hence, $MARTX_{Vc}$ is a multifunctional toxin that causes disassembly of the cell cytoskeleton by both direct and indirect mechanisms.

AUTOPROCESSING OF MARTX TOXINS

The only other RTX toxin that is multifunctional is *B. pertussis* ACT, which has both hemolytic and adenylate cyclase activities (20). Similar to ACT, it is likely that the $MARTX_{Vc}$ toxin self-inserts into the eukaryotic membrane and translocates its catalytic domains to the cytosol, where it can access the host target proteins (31). However, unlike ACT, which requires access only to intracellular ATP directly below the membrane to function as an adenylate cyclase, the activities associated with $MARTX_{Vc}$ suggest that the ACD and RID need to be released freely to the cytosol to access targets. Therefore, it was predicted that $MARTX_{Vc}$ would undergo proteolytic cleavage during translocation into host cells (31). A domain located at aa 3420 to 3526 has been shown to be an autocatalytic cysteine protease domain (CPD) whose activity is essential to the toxic action of $MARTX_{Vc}$ (30). Since autocleavage would not be expected until after translocation, it is not surprising that activation of the protease in vitro required addition of eukaryotic cell cytosol preparations. It was subsequently shown that binding, but not hydrolysis, of GTP was sufficient to stimulate processing, indicating that processing occurs after transit of the toxin to the eukaryotic cytoplasm with high GTP concentrations (30).

The CPD located upstream of the C repeats is conserved in all of the MARTX toxins (Fig. 2), indicating that autoprocessing is a defining feature of this family of toxins. Detailed alignments show that all MARTX toxin CPDs include the histidine and cysteine that comprise the catalytic dyad, indicating that all of them could be enzymatically functional (30). Thus, it seems that all MARTX toxins have the propensity for autocatalytic cleavage.

ACTIVITY DOMAINS CARRIED BY OTHER MARTX TOXINS

As shown in Fig. 2, the MARTX toxins have large sections where sequences are conserved at the N and C termini, but the protein sequences are quite distinct in the central regions. For $MARTX_{Vc}$, domains associated with actin cross-linking (ACD) and Rho GTPase inactivation (RID) are located within this central region. Thus, the sequence divergence of toxins in the central regions indicates that each MARTX toxin has different toxic activities and does not necessarily cause cell rounding. The conservation of the CPD further indicates that these domains could be delivered to the cytosol by autoproteolytic cleavage.

Overall, the toxins appear to have a mosaic structure in the central region, in which 10 different domains are assorted among the individual toxins (Fig. 2). For example, the ACD is shared by the $MARTX_{Vc}$ and $MARTX_{Ah}$ toxins but is absent from other toxins. By contrast, the RID is found in $MARTX_{Vc}$, $MARTX_{Vv}$, and both toxins from *Xenorhabdus* spp. $MARTX_{Vc}$

clearly has an additional domain shared with five other toxins that has yet to be characterized. A conserved domain search revealed that this uncharacterized domain is a member of the alpha-beta (α/β) hydrolase protein family (25). These proteins share a common stable tertiary structure for presentation of a catalytic triad, although the enzymatic activities vary and the enzymes include proteases, elastases, lipases, and esterases (17).

Even though $MARTX_{Vc}$ toxins encoded in nine sequenced *V. cholerae* genomes are nearly identical, the putative MARTX toxin from the environmental isolate RC385 ($MARTX_{VR}$) is entirely distinct. As sequenced, the toxin gene has a frameshift mutation, but there may be other isolates in which the gene is intact. The putative full-length toxin could have three toxic activities: two domains having unknown functions (DUF1 and DUF2) shared with $MARTX_{Ye}$ and another domain that is conserved with the adenylate cyclase domain from *Pseudomonas aeruginosa* type III secretion effector ExoY, indicating that this toxin is likely an adenylate cyclase toxin.

The large size of $MARTX_{Vv}$ is due to its five putative activity domains. In addition to RID and the α/β hydrolase, this toxin has a domain with an unknown function (DUF3) shared with the *Xenorhabus* sp. toxins. The fourth domain is shared with four other MARTX toxins and is also similar to a domain of the *P. luminescens* Mcf toxins (makes caterpillars floppy). Mcf1 has been shown to induce apoptosis of mammalian cells, although the domain of the toxin that induces apoptosis has not been mapped to a specific region (11, 35). The fifth domain of $MARTX_{Vv}$ is shared with three other MARTX toxins and is also similar to a portion of the *Pasteurella* mitogenic toxin (PMT). The structure of the C terminus of PMT has recently been solved and shown to fold into three domains (19). The region conserved in the MARTX toxins corresponds to C1, a lipid membrane localization domain, plus C2, a domain with two α/β hydrolase fold subdomains, suggesting that this domain could have two catalytic activities (19). Although the mitogenic activity of PMT is localized to the C terminus (6, 28), a thiol protease found within C3 is proposed to be the catalytic region (19), Thus, potential activities carried by the C1 and C2 domains and thus MARTX toxins are currently unknown. However, even though the thiol protease of PMT is not related to the MARTX CPD, analogy to studies with $MARTX_{Vc}$ CPD could suggest that the thiol protease is required for autoprocessing of PMT to release C1 and C2 to the cytosol, indicating that the mitogenic activity of PMT could be conserved in the MARTX toxins. Recently, mutations in the *rtxA* gene of *V. vulnificus* have been characterized, and it has been shown that $MARTX_{Vv}$ is a cytolysin that causes lysis of epithelial cells within 90 to 180 min after addition of bacteria (21, 23). Thus, one of the five activity domains of $MARTX_{Vv}$ could be an enzyme that perturbs membrane integrity, although the region of the toxin responsible for cell lysis has yet to be identified.

The last activity domain of the MARTX toxins is a domain with an unknown function (DUF4) carried by *Vibrio splendidus* MARTX and the four $MARTX_{Pl}$ toxins. This domain is apparently the only activity domain of $MARTX_{Pl1}$, suggesting that this toxin may not be multifunctional. However, this domain is over 500 aa long and may itself have several activities that always recombine into new toxins as a single domain. Thus, it is possible that all the MARTX toxins are indeed multifunctional and that one or more activities are associated with each identified domain.

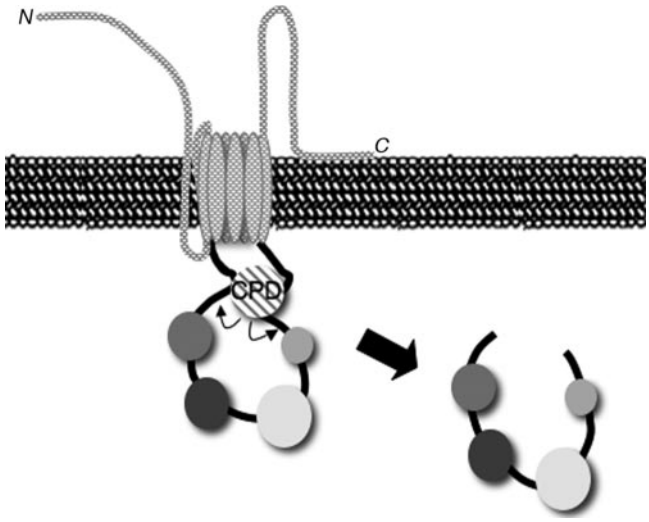

FIG. 4. Comprehensive model for delivery of MARTX toxin activity domains as detailed in the text.

COMPREHENSIVE MODEL FOR MARTX TOXINS

From the analysis presented here, it is evident that $MARTX_{Vc}$ typifies a new family of RTX toxins of human, marine, and insect pathogens. These toxins share numerous characteristics, including three sets of glycine-rich repeats and potentially common mechanisms of maturation, secretion, and translocation. By contrast, comparison of the deduced amino acid sequences suggests that these toxins have different cellular activities that are advantageous to the particular bacteria. As a comprehensive model, it is proposed that the N- and C-terminal repeat regions form a structure within the eukaryotic cytosolic membrane that is necessary to translocate centrally located activity domains across the membrane. Upon transfer of the CPD into the cytosol, the toxin would be autoprocessed, releasing activity domains to the cytosol, where they could move freely through the cell to access cellular targets (Fig. 4). The repertoire of activities carried by the multifunctional toxins would ultimately be dictated by the selection of activity domains that they carry.

As such, this new MARTX family of toxins is of biochemical interest because of their unusual structural properties and unknown cytopathic effects, and this is important since members of this family are likely to be involved in pathogenesis in many different kinds of living organisms, including mammals, fish, amphibians, nematodes, and insects.

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