Type VI secretion system translocates a phage tail spike-like protein into target cells where it cross-links actin

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Genes encoding type VI secretion systems (T6SS) are widely distributed in pathogenic Gram-negative bacterial species. In *Vibrio cholerae*, T6SS have been found to secrete three related proteins extracellularly, VgrG-1, VgrG-2, and VgrG-3. VgrG-1 can covalently cross-link actin *in vitro*, and this activity was used to demonstrate that *V. cholerae* can translocate VgrG-1 into macrophages by a T6SS-dependent mechanism. Protein structure search algorithms predict that VgrG-related proteins likely assemble into a trimeric complex that is analogous to that formed by the two trimeric proteins gp27 and gp5 that make up the baseplate "tail spike" of *Escherichia coli* bacteriophage T4. VgrG-1 was shown to interact with itself, VgrG-2, and VgrG-3, suggesting that such a complex does form. Because the phage tail spike protein complex acts as a membrane-penetrating structure as well as a conduit for the passage of DNA into phage-infected cells, we propose that the VgrG components of the T6SS apparatus may assemble a "cell-puncturing device" analogous to phage tail spikes to deliver effector protein domains through membranes of target host cells.

bacteriophage | cytotoxicity | *Vibrio cholerae* | virulence

Secretion of proteins is one means by which microbes influence their extracellular milieu, which, in the case of pathogenic microbes, includes target host cells (for review see ref. 1). During pathogenesis, bacteria can affect host cells through the export of toxins and enzymes that alter cellular function or through the assembly of extracellular structures such as pili and adhesins that promote adherence to host cell surfaces. Bacteria have also evolved specialized surface structures that deliver proteins and nucleic acids directly into target cells, an export process referred to as translocation. Gram-negative bacterial pathogens use at least six distinct extracellular protein secretion systems (referred to as type I–VI, or T1SS–T6SS) to export proteins through their multilayer cell envelope and, in some cases, into the host target cells (1–9). These secretion systems are distinguished in part by the conserved structural components that define them but also by the characteristics of their substrates and the path that these substrates take during the export process. For example, type T2SS and T5SS systems recognize protein substrates that have been transported through the inner membrane and then transport these substrates through the periplasm and across the outer membrane. T1SS, T3SS, and T4SS systems transport cytoplasmic protein substrates directly through both the inner and outer membrane (for review see ref. 1).

Less is known about the mechanism of protein transport by the newly described T6SS (8, 9). T6SS gene clusters are highly conserved and are present in one or more copies in many other pathogenic Gram-negative bacterial species, including *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Yersinia pestis*, *Escherichia coli*, *Salmonella enterica*, *Agrobacterium tumefaciens*, *Rhizobium leguminosarum*, *Francisella tularensis*, *Burkholderia mallei*, and *Edwardsiella* species (8, 10–18). T6SS genes have been implicated in virulence-related processes in several of these organisms (14, 18–23).

Using the social amoeba *Dictyostelium discoideum* as a host model of infection, we identified T6SS as a crucial virulence determinant of *V. cholerae* (7). In *V. cholerae*, inactivation of type VI genes (termed *vas* loci) resulted in an extracellular protein secretion defect and loss of cytotoxicity toward amoebae and J774 macrophages (7). The *vas* gene cluster-encoded T6SS mediates the extracellular secretion of four distinct proteins (Hcp, VgrG-1, VgrG-2, and VgrG-3). Mutations in the *hcp* or *vgrG-2* genes attenuate cytotoxicity and block secretion of the other T6SS protein substrates. The proteins exported by the *V. cholerae* T6SS lack amino-terminal hydrophobic signal sequences and appear in culture supernatant as unprocessed polypeptides. As in T3SS and T4SS, inactivation of T6SS components results in accumulation of substrates inside bacterial cells distributed between the cytosol and the periplasm depending on mutation and system (7, 8, 12).

Secretion of Hcp is the hallmark of a functional T6SS in many bacterial species. Other organisms that carry T6SS gene clusters typically regulate secretion of Hcp orthologs. For example, certain regulatory mutants of *P. aeruginosa* secretes an Hcp homolog (8). Cystic fibrosis (CF) patients with chronic *P. aeruginosa* lung infections will produce antibodies to Hcp after several years of infection, suggesting that the T6SS is expressed only after adaptation of *P. aeruginosa* to the chronic inflammatory environment of the CF lung (8). *B. mallei* tightly regulates a T6SS that secretes an Hcp ortholog, and mutations that block its secretion are profoundly attenuated in a hamster model (18). An Hcp ortholog is also secreted by the T6SS of enteroaggregative *E. coli*, but its role in pathogenesis is not known (10). The fish pathogen *Edwardsiella tarda* secretes the Hcp homolog EvpC, and mutations in genes corresponding to those present in the *V. cholerae* T6SS cluster fail to multiply inside phagocytic cells from fish (18).

Hcp genes are often found embedded in T6SS gene clusters and adjacent to *vgrG* genes (7, 8, 10–18). The close proximity of *hcp* and *vgrG* genes to other T6SS genes, suggests that VgrG and Hcp may be preferred substrates or essential components of the T6SS apparatus. *V. cholerae* contains three genes that encode VgrG proteins, *vgrG-1*, *vgrG-2*, and *vgrG-3*, and inactivation of *hcp* blocks secretion of all three of these proteins (7). Inactivation of VgrG-2 blocks secretion of Hcp, VgrG-1, and VgrG-3 and attenuates *V. cholerae* for cytotoxicity toward *Dictyostelium* amoebae and J774 macrophages (7). Thus, Hcp and VgrG-2 may

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Abbreviations: VAS, virulence associated secretion; T6SS, type VI secretion.

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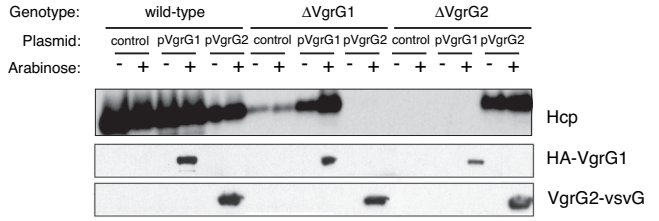

Fig. 1. Hcp export requires the presence of VgrG-1 and VgrG-2. Plasmids allowing the inducible expression of HA-tagged VgrG-1 (pVgrG1), vsvG-tagged VgrG-2 (pVgrG2), or plasmid control (control) were introduced into wild-type *V. cholerae*, or isogenic mutants lacking VgrG-1 (ΔvgrG1) or VgrG-2 (ΔvgrG2). Culture supernatants from cells grown in liquid broth under inducing (+) and noninducing (−) conditions were concentrated and separated by SDS/PAGE; Hcp was visualized by Western blot analysis with Hcp antiserum. To guarantee plasmid-borne expression of HA-tagged VgrG-1 and vsvG-tagged VgrG-2, cell extracts were separated by SDS/PAGE for immunoblotting with HA and vsvG antisera, respectively.

contribute to the function of T6S apparatus despite the fact that they also appear to be transported substrates of the system.

In this article, we present evidence that the *V. cholerae* T6SS likely builds a structure that is predicted to be similar to the tail spike protein complex of the *E. coli* bacteriophage T4 (24). One of the proteins predicted to be in the T6SS complex (VgrG-1) encodes an enzyme that covalently cross-links host cell actin, and we show here that it is responsible for T6SS-dependent macrophage cytotoxicity. By analogy to phage tail spikes, the predicted VgrG trimeric complex may serve the purpose of puncturing host membranes as well as serving as a channel for export of macromolecules out of the bacterial cell and into a target cell.

Results

Secretion of Hcp Requires VgrG-1 and VgrG-2. To understand the various roles that the *vgrG* and *hcp* genes play in T6SS-dependent secretion of Hcp and virulence toward eukaryotic cells, we performed a series of genetic, biochemical, and cell-biological analyses. The role of *vgrG-1* and *vgrG-3* in these phenotypes had not previously been determined. An in-frame deletion of *vgrG-3* showed no defect in Hcp secretion, nor was this mutant any less virulent toward amoebae than wild-type V52 (data not shown). However, mutants carrying in-frame deletions of *vgrG-1* or *vgrG-2* were unable to secrete epitope tagged versions of VgrG-1 and VgrG-2 (Fig. 1). These mutants were also unable to secrete wild type levels of Hcp (Fig. 1). These defects were most efficiently complemented by arabinose-inducible expression of the homologous gene but not the heterologous gene, suggesting that both VgrG-1 and VgrG-2 were essential components of the T6SS. Mutants defective in *vgrG-1* and *vgrG-2* also showed no virulence toward amoebae and mammalian macrophage cell lines, such as J774 and RAW 264.7 cells (data not shown).

VgrG-1 Cross-Links Actin. Although all VgrG proteins are related to each other through several conserved domains (see below), VgrG-1 is unique in that it carries a C-terminal extension of 446-aa residues that shares extensive homology to the actin cross-linking domain (ACD) of the *V. cholerae* RtxA toxin (25) [see supporting information (SI) Fig. 6]. Indeed, ectopic expression of the ACD from VgrG-1 inside eukaryotic cells was reported to cause cell rounding and covalent cross-linking of actin (25).

To confirm that the *vgrG-1* gene of strain V52 encodes a protein with actin cross-linking enzymatic activity, we developed an *in vitro* assay based largely on the conditions established by Cordero *et al.* (26). A hexahistidine-tagged version of VgrG-1 was expressed and purified from *E. coli* by using Ni-affinity chromatography (Fig. 2*A*). This recombinant protein was mixed with purified monomeric G-actin, cytoplasmic extracts of *Dictyostelium* amoebae, or cytoplasmic extracts of murine RAW 264.7 macrophages. Anti-actin Western blot analysis (Fig. 2*B*) showed that VgrG-1 catalyzed cross-linking of actin in all three substrate sources by a mechanism that depended on Mg-ATP. Thus, consistent with its role as an effector protein of the *V. cholerae* T6SS, VgrG-1 carries an active enzymatic domain that could act on essential eukaryotic cytoskeletal protein.

To investigate whether the actin cross-linking activity of VgrG-1 is responsible for the T6SS-dependent cell rounding phenotype we had observed (7), we analyzed the level of cross-linked actin in J774 cells that had been exposed to *V. cholerae* V52 and its derivatives (Fig. 3). To differentiate between VgrG-1- and RtxA-mediated actin cross-linking, we an-

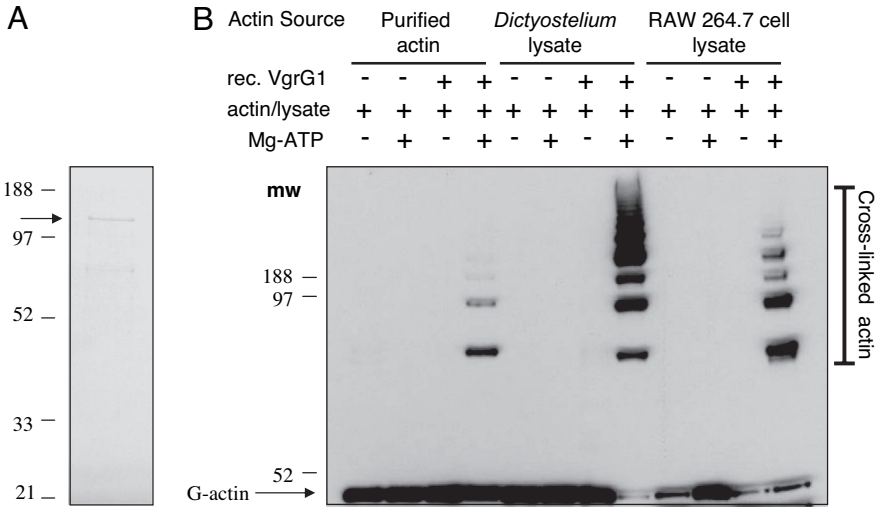

Fig. 2. Recombinant VgrG-1 cross-links actin in an ATP-dependent fashion. (*A*) Hexahistidine-tagged VgrG-1 was purified on a nickel-NTA column and separated by SDS/PAGE (see arrow). (*B*) Recombinant VgrG-1 was incubated at 37°C for 2 h with purified rabbit skeletal monomeric G-actin (Cytoskeleton, Denver, CO) or crude lysates from *Dictyostelium* and RAW 264.7. Reactions were supplemented with 2 mM Mg-ATP as indicated. Reaction mixtures were separated by SDS/PAGE, and actin was visualized by Western blot analysis with actin antiserum.

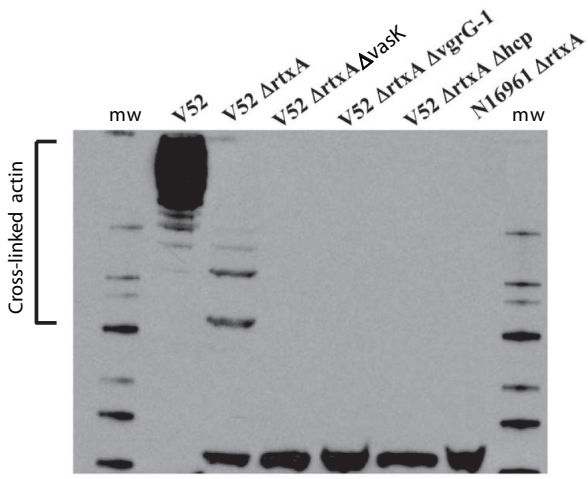

Fig. 3. VgrG-1-mediated cross-linking of the host actin cytoskeleton. Cultured J774 cells were infected for 2 h with indicated *V. cholerae* strains at a multiplicity of infection (MOI) of 10. Extracts of infected cells were separated by SDS/PAGE for immunoblotting with actin antiserum.

alyzed the appropriate single, double, and triple mutants of *rtxA*, *vgrG-1*, *hcp*, and *vasK* (an *icmF* ortholog) required for T6SS-mediated cytotoxicity (7). After J774 cells were incubated with various *V. cholerae* strains, cell extracts were prepared in SDS sample buffer and analyzed by Western blotting with anti-actin antibody (Fig. 3). *V. cholerae* strain V52 induced massive cross-linking of cytosolic actin in J774 cells within 2 h of incubation. The level of actin cross-linking was reduced but not eliminated by inactivation of *rtxA*. However, no actin cross-linking could be observed in extracts from J774 cells treated with mutants lacking both *rtxA* and either *vasK* or *vgrG-1*. A triple mutant deficient in *rtxA*, *hcp-1*, and *hcp-2* was also completely defective in actin cross-linking (Fig. 3). Interestingly, an *rtxA* mutant of the 7th pandemic El Tor O1 strain N16961 did not cross-link actin either (Fig. 3); this result supports published data that El Tor O1 strains have an undefined defect in the expression of the T6SS-dependent J774 cytotoxicity phenotype (7). Together, these data indicate that the T6SS of *V. cholerae* V52 is apparently able to transfer the ACD of VgrG-1 into the cytosol of J774 cells where it can reach its substrate, G-actin. The transfer of the ACD of VgrG-1 into target cells depends on Hcp and VasK. Because VgrG-1 plays a role both as an effector and as a functional part of the T6S apparatus, we cannot say whether a *vgrG-1* mutant's defect in actin cross-linking reflects disruption of the entire T6S apparatus or simply loss of a critical T6SS effector.

VgrG Proteins Share Structural Features with Phage Tail Spike Proteins. Having established that VgrG-1 can be transported into target eukaryotic cells by the T6SS of *V. cholerae*, we hoped to learn more about this protein's predicted molecular structure. A bioinformatic analysis was performed on the predicted VgrG-1 protein sequence as well as other related proteins present in public databases. As shown schematically in Fig. 4*A*, the three *V. cholerae* VgrG proteins each contain two highly homologous N-terminal domains and various C-terminal extensions, such as the ACD domain of VgrG-1. By using various programs (PSI-Blast, PSIPRED, Robetta, and HHPRED (27–31), the two N-terminal domains were consistently identified as protein domains that resemble the gp44 protein of bacteriophage Mu (32) and the gp5 protein of bacteriophage T4 (24) with highly significant probabilities (e.g., PSI-BLAST: *e* value <*5*e-172 and PSIPRED: *e* value <*1*e-8). Both these proteins are components

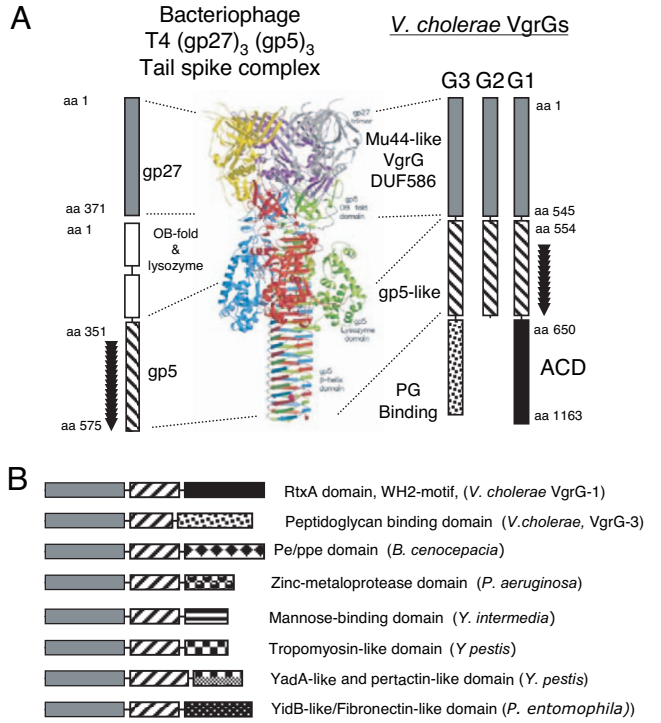

Fig. 4. VgrGs share structural features with the phage tail of bacteriophage T4. (*A*) *V. cholera* VgrGs are a fusion of the phage tail proteins gp27 (gray) and gp5 (hatched), omitting the OB-fold and lysozyme domains (white) of the phage gp5 protein. The gp5 domain consists of a β-helix repeat (black arrows) that forms the cell-puncturing ''needle'' of the phage tail. VgrG-1 and VgrG-3 contain a C-terminal actin-cross-linking domain (ACD) and peptidoglycan (PG)-binding domain, respectively. Amino acid numbers are designated for gp27, gp5, and VgrG-1. Crystal structure for the phage tail spike (gp27 and gp5) are shown for reference (24) (adapted by permission from Macmillan Publishers Ltd: *Nature*, 31 January 2002). (*B*) Graphical representation of a subset of the VgrG-family members with extended C-terminal domains. These proteins consist of a gp27-like domain (solid black), gp5-like domain (hatched) and different C-terminal extensions (bars with various patterns). On the right is shown the predicted structural similarities of the C-terminal domains identified by the PSIPRED protein structure prediction server (http://bioinf.cs.ucl.ac.uk/psipred/). Protein structures schematically shown from top to bottom have the following accession numbers: AAF94573, AAF96037, ZP_00981404, NP_248953, ZP_00832947, CAC92964, YP_652030, and YP_606302. A more extensive list of VgrG orthologs with extended C termini together with PSIPRED predicted structural similarities can be found in SI Table 1.

of the bacteriophage tail spike complexes, a device that is known to insert through the bacterial outer membrane during phage infection (24, 33). PSI-PRED analysis (29) also predicts that Mu gp44 is clearly a structural ortholog of tail spike protein gp27 of phage T4 (32, 24). As schematically shown in Fig. 4*A*, the tail spike of bacteriophage T4 can be represented as a $(gp27)_3(gp5)_3$ dimeric complex of these two trimer proteins (Fig. 4*A*). Thus, two homotrimers of a gp27-like protein and gp5-like protein assemble "hat on head" to form the long extended tail spikes that are common to the base plates of virtually all tailed bacteriophages belonging to the order *Caudovirales* and family *Myoviridae* (34).

Although two phage genes encode gp27 and gp5, the two structurally corresponding protein domains in VgrGs are fused into a single polypeptide (Fig. 4*A*). Relative to gp5, all VgrG proteins also lack two domains, the OB fold and lysozyme domains, which exist as N-terminal extensions on the gp5 repeat domain. In the T4 tail spike assembly, the lysozyme domain axially extends out and away from the rod like gp5/gp27 structure, whereas the OB fold domain forms another trimeric

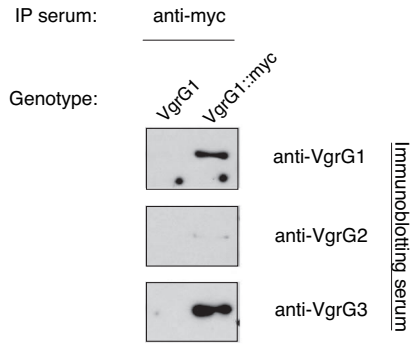

Fig. 5. Immunoprecipitation of VgrG-1-myc. *V. cholerae* strains expressing either wild-type VgrG-1 or a VgrG-1::myc tagged protein were grown to midlogarithmic growth phase. Culture supernatants were filtered and Myc antisera conjugated to agarose beads were added to pull-down VgrG-1::myc and any interacting proteins. Resulting precipitates were separated by SDS/PAGE for immunoblotting with antisera specific for VgrG-1, VgrG-2, and VgrG-3. The bands corresponding to the size of VgrG-1, VgrG-2, and VgrG-3 were identified only in immunoprecipitations from supernatants derived from the strain expressing VgrG-1-myc.

structure that makes up the gp5 trimer "head" on which the trimeric gp27 "hat" sits (Fig. 4*A*). Although VgrGs lack the OB fold and lysozyme domains, the two other critical trimer structural characteristics of the tail spike proteins are maintained in the three fused VgrG proteins from *V. cholerae*. These include the gp5 repeat domain, which forms a triple stranded β-helix in gp5 (see below) and the gp27 domain, which forms the trimeric ring that sits on top of the $(gp5)_3$ assembly (24).

Besides the gp27-like domain, the other striking domain that is apparent in all *V. cholerae* VgrGs is a region that contains 12-aa repeats of eight residues in length that correspond to a region of 17 repeats of eight residues in length located at the C terminus of gp5 (Fig. 4*A*). In the case of T4 phage, these repeats assemble into a triple stranded β-helix forming an equilateral triangular prism that is highly stable and resists dissociation in 10% SDS and 2 M guanidinium HCl (24). It is this needle-like tube that can be seen protruding from the bottom of the T4 phage base plate as the point of the tail spike (34). VgrG members all contain this corresponding repeat region, and PSI-PRED analysis (29) predicts that these repeats should assemble into a triple-stranded β-helix of 12 turns in length (SI Fig. 7). Furthermore, the assembly of this trimeric β-helical needle should be driven or stabilized by the predicted trimeric interactions of the gp27-like domain to which the VgrG repeat region is directly attached. Together, these data suggest that VgrG proteins assemble into homotrimers, or heterotrimers, depending on the stochiometry of expression of VgrG-1, VgrG-2, and VgrG-3.

VgrG-1 Is Part of a Larger Complex. To test whether VgrG multimers form, we performed immuno affinity "pull-down" experiments, in which different VgrG proteins were differentiated from one another by using specific antisera. Culture supernatant fluids from *V. cholerae* strains expressing either wild-type VgrG-1 or a VgrG-1::myc-tagged protein from single copies of chromosomally located genes were subjected to immunoprecipitation using agar beads conjugated with anti-Myc-tagged antibody. The resulting precipitates were separated by SDS/PAGE and analyzed by immunoblotting using antisera specific for VgrG-1, VgrG-2, and VgrG-3; these sera were developed against unique peptides present in each VgrG protein to avoid immunological cross-reactions. As shown in Fig. 5, bands corresponding to the size of VgrG-1, VgrG-2, and VgrG-3 were identified only in immunoprecipitations from supernatants derived from the strain expressing VgrG-1-myc. These data indicate that VgrG-2 and VgrG-3 interact with VgrG-1-myc in the supernatant fraction. We have also performed immunoprecipitations from whole-cell extracts expressing different tagged versions of VgrG proteins and found that Myc-tagged VgrG-1 could pull down HA-tagged-VgrG-1, VsvG-tagged VgrG-2, and HA-tagged VgrG-3 (data not shown). Thus, VgrG proteins can interact in various combinations, suggesting they may be capable of forming homotrimeric as well as heterotrimeric complexes.

Functional Predictions for VgrG Proteins Encoded by Other Gram-Negative Bacteria. Given the wide distribution of T6SS gene clusters (7, 8, 10–18) and the importance of the ACD domain in the host effector function of VgrG-1, we reasoned that other VgrG family members should exist that also contain extended C termini involved in T6SS effector function. The shortest of the *V. cholerae* VgrG proteins (VgrG-2) was used as a "core" VgrG to search the National Center for Biotechnology Information (NCBI) database for VgrG-like proteins (SI Fig. 8). This search yielded >770 proteins with amino acid sequences that were similar to VgrG-2. Using VgrG-2 as the ruler, we omitted proteins from further evaluation that were <750 aa in length. The resultant 98 predicted proteins (SI Table 1) had a similar overall domain architecture to that of VgrG-1 and VgrG-3; each contained an N-terminal Mu44/gp27-like domain, followed by a gp5-like repeat domain and an extended C-terminal domain that corresponded in location to the ACD of VgrG-1. We call such proteins "evolved VgrG" proteins because of the extensive variation in the C-terminal domains. Evolved VgrG proteins were encoded by many pathogenic genera, including *Burkholderia*, *Pseudomonas*, *Vibrio*, and *Yersinia* (Fig. 4*B*). Of note, all species encoding a longer evolved VgrG ortholog also encoded a shorter "core" version (similar to VgrG-2). Most of these species also encoded an intact cluster of T6SS genes and at least one *hcp*-related gene (A.T.R. and J.J.M., unpublished observations). These data are consistent with the hypothesis that these longer VgrG proteins are T6SS effectors but may assemble with other VgrG-2-like proteins to form heterotrimeric complexes.

In an attempt to identify novel domains represented by the extended C-terminal domains of evolved VgrG proteins, we performed PSIPRED analysis (29) to compare these C-terminal protein domains with proteins for which solved x-ray crystal structures are available (i.e., fold-recognition analysis). This analysis is preliminary at best and suffers from multiple limitations, most notably the inability to perform comparisons with proteins that do not yet have solved crystal structures. For example, although VgrG-1 is highly homologous to the RtxA toxin of *V. cholerae* in their shared ACD domains (25), PSIPRED predictions did not identify this homology because the ACD domain of RtxA has not yet been crystallized. Nonetheless, we performed this analysis to understand some of the diversity in function that might be represented by the C-terminal domains of evolved VgrG proteins (SI Table 1).

Analysis of VgrG-3 indicates that this protein contains a peptidoglycan-binding domain (*e*-value: 5*e*-7) located in its C terminus. However, it is unclear whether this C-terminal extension plays a role in anchoring the T6SS apparatus to the bacterial cell envelope or whether it predicts an anti-bacterial functionality for this particular VgrG. The C-terminal extensions of evolved VgrG proteins included domains that were highly homologous to other virulence proteins including the adhesins pertactin of *Bordetella* (35) and YadA of *Yersinia* (36) as well virulence proteins of unknown function such as the pe/ppe proteins of *Mycobacterium* (37). Other VgrG C-terminal extensions showed significant similarities to mannose-binding proteins or enzymes such as proteases. One group of eight VgrGs from *Yersinia* carried C-terminal extensions that were highly similar to

eukaryotic tropomyosin, a protein that stabilizes actin filaments (38) and thus might be involved in manipulating target cell cytoskeleton.

Discussion

Non-01/non-O139 strains of *V. cholerae* represent a diverse group of pathogenic strains whose virulence properties are largely uncharacterized (39, 40). In previous work, we used a *Dictyostelium* model to identify T6SS as a bacterial virulence determinant for strain V52, a pathogenic 037 strain (7). The data presented here provide evidence that the T6SS of *V. cholerae* uses VgrG-1 to translocate effector proteins and to cross-link actin of infected J774 macrophages. The secretion of VgrG-1 requires the closely related protein VgrG-2 but not VgrG-3. We also used structural prediction programs to analyze the VgrG proteins and made a remarkable observation. Most VgrG proteins, which include hundreds of examples in the protein database, are predicted to have a structure related to two trimeric proteins, gp27 and gp5, which together assemble into the tail spike complex of bacteriophage T4 (24, 33, 34). In support of this observation, we show that VgrG proteins do interact with each other in immunoprecipitation assays. Thus, VgrG proteins may form a trimeric complex analogous to the $(gp27)_3(gp5)_3$ complex that defines the tail spike membrane-puncturing device of phage T4 (24).

The trimer VgrG complexes that we envision might play multiple roles. They could serve as part of a channel for transport of T6SS substrates such as Hcp and VgrG proteins out of the bacterial cell. In this case the β-helical needle formed by three VgrGs could serve to puncture the outer membrane of the bacterial cell; this is topologically the exact opposite (from the inside out) to the membrane puncturing event that the T4 spike complex is thought to perform (from outside in) (24, 33, 34). The C terminus β-helical needle formed by VgrG trimers might also serve as an extracellular "translocon" (1) whose role is to puncture target host cell membranes and deliver effector domains such as VgrG-1's ACD domain into the target cell cytosol. The "hollow cylinder" formed by trimeric gp27 in the T4 $(gp27)_3(gp5)_3$ is 30 Å (24, 33) and large enough to easily accommodate a α-helix of 12 Å in width. Alternatively, a channel might also be formed by other components of the T6SS apparatus with the VgrG complex simply serving as the initial membrane-puncturing component. In this regard, the atomic structure of the Hcp ortholog of *P. aeruginosa* was reported to be a six-membered ring with a 40-Å central channel (8). The crystal lattice of Hcp suggests that this protein could stack to form long tubes that might correspond to a transport channel for T6SS substrates (8). Thus, it is possible that Hcp rings or tubes might interact with the gp27-like domain of the hypothetical trimeric VgrG complex. The diameter of the Hcp hexamer is sufficient to accommodate the predicted channel that would be formed by the VgrG trimer (i.e., 30 Å), and together, these might define an extracellular T6SS translocon.

It is also worth noting that a ClpB-related protein, ClpV, has been implicated in the assembly and function of the T6SS apparatus in *P. aeruginosa* (8). Members of the ClpB family of proteins assemble into hexameric, ring-shaped structures and then hydrolyze ATP to provide energy for insertion of protein substrates into their central channel (41). Thus, VgrG proteins represent the third T6SS-associated protein family predicted to form a protein complex that displays a central channel. The central channels of the hexameric Hcp and ClpB oligomers might align with the proposed channel formed by VgrG trimeric complexes in such a way to provide a conduit for T6SS-dependent translocation of proteins out of and between cells. It is interesting to note that the T4SS of *Legionella* is capable of transporting both proteins and DNA between cells (42). Future work will seek to demonstrate that T6SS are machines that have evolved to translocate effector proteins (and perhaps nucleic acids) between cells in ways that enhance the virulence or evolutionary fitness of T6SS+ bacterial species. The large family of "evolved VgrG"-related proteins described here represent interesting candidates for such T6SS effectors.

Materials and Methods

Strains, Plasmids, and Culture Conditions. *Dictyostelium discoideum* strain AX3 was used in all experiments. AX3 was grown in liquid HL/5 cultures or in lawns of *Klebsiella aerogenes* on SM/5 plates, as described by Sussman (43). *V. cholerae* O37 serogroup strain V52 or derivatives were used in all experiments described. *E. coli* strains DH5α-λpir and BL21 (DE3) were used for cloning and protein expression, respectively. All bacterial strains were grown in Luria broth (LB). J774 and RAW 264.7 cells were obtained from American Type Culture Collection (Manassas, VA). In-frame gene deletions and chromosomal C-terminal VgrG-1::myc–epitope fusions were generated by using the method of Skorupski and Taylor (44). Plasmid pVgrG-1 was constructed for arabinose-inducible expression of VgrG-1 tagged with an amino-terminal HA (influenza hemagglutin peptide) epitope. HA-tagged *vgrG-1* was PCR-amplified from chromosomal *V. cholerae* (strain V52) DNA by using primers 5′-HA-V1 (GAATTCACC ATG TAT CCT TAT GAT GTT CCT GAT TAT GCA GCG ACA TTA) and 3′-V1 (TCTAGATTAAGCAATAATGCGTTGCCA). The resulting PCR product was digested with EcoRI and XbaI, and subcloned into plasmid pBAD24. Plasmid pVgrG-1, which allows expression of VgrG-2 tagged with a carboxyl-terminal VsvG peptide, was essentially constructed as pVgrG-2, except that primers 5′-vgrG-2 (GAATTCACC ATG GCG ACA TTA GCG TAC) and 3′-vsvG-vgrG-2 (TCTAGA TTA TTT TCC TAA TCT ATT CAT TTC AAT ATC TGT ATA ATT TCC CTT GGC CTC TTC) were used.

Immunoprecipitation and Western Blots. Protein blotting techniques were performed as described previously (45). Immunoprecipitation of Myc-tagged VgrG-1 with Myc-antiserum (Bethyl Laboratories, Montgomery, TX) was carried out as described (46).

***In Vivo* Actin Cross-Linking.** J774 cells were seeded into six-well tissue culture plates at a density of 10^6 cells per well. After 16-h incubation at 37°C, cells were infected with various *V. cholerae* strains, all of which are deficient in *hlyA* and *hapA*. The 2-h infections were performed at a multiplicity of infection of 10. Cells were harvested and resuspended in 50 μl of sample buffer. Ten microliters of each sample was analyzed by Western blot using actin antiserum (Sigma–Aldrich, St. Louis, MO).

***In Vitro* Actin Cross-Linking.** For the isolation of hexahistidine-tagged VgrG-1 (His_6-VgrG-1), *vgrG-1* was moved into plasmid pDEST17-His_6 by using Gateway technology (Invitrogen, Carlsbad, CA). Recombinant His_6-VgrG-1 was expressed in *E. coli* strain BL21 (DE3) and purified on a nickel-NTA column with the QIAexpressionist kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Recombinant VgrG-1 was incubated at 37°C for 2 h with purified rabbit skeletal monomeric G-actin (Cytoskeleton) or crude lysates from *Dictyostelium* and RAW cells. Reactions were supplemented with 2 mM Mg-ATP as indicated. Reaction mixtures were separated by SDS/PAGE, and actin was visualized by Western blot analysis with actin antiserum (Santa Cruz Biotechnology, Santa Cruz, CA).

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