CALCIUM IS ESSENTIAL FOR THE MAJOR PSEUDOPILIN IN THE TYPE 2 SECRETION SYSTEM*

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The pseudopilus is a key feature of the type 2 secretion system (T2SS) and is made up of multiple pseudopilins which are similar in fold to the type IV pilins. However, pilins have disulfide bridges whereas the major pseudopilins of T2SS do not. A key question is therefore how the pseudopilins, and in particular the most abundant “major” pseudopilin GspG, obtain sufficient stability to perform their function. Crystal structures of Vibrio cholerae, Vibrio vulnificus and enterohaemorrhagic Escherichia coli (EHEC) GspG were elucidated and all show a calcium ion bound at the same site. Conservation of the calcium ligands fully supports the suggestion that calcium ion binding by the major pseudopilin is essential for the T2SS. Functional studies of GspG with mutated calcium ion coordinating ligands were performed to investigate this hypothesis and show that in vivo protease secretion by the T2SS is severely impaired. Taking all evidence together this allows the conclusion that, in complete contrast to the situation in the type 4 pilus system homologs, in the T2SS the major protein component of the central pseudopilus is dependent on calcium ions for activity.

In Gram-negative bacteria, the type 2 secretion system (T2SS) is used for the secretion of several important proteins across the outer membrane(1). The T2SS is also called the terminal branch of the general secretory pathway (Gsp)(2) and, in Vibrio species, the extracellular protein secretion (Eps) apparatus(3). This sophisticated multi-protein machinery spans both the inner and the outer membrane of Gram-negative bacteria and contains 11 to 15 different proteins. The T2SS consists of three major subassemblies (4-9): (i) the outer membrane complex comprising mainly the crucial multi-subunit secretin GspD; (ii) the pseudopilus, which consists of one major and several minor pseudopilins; and, (iii) an inner membrane platform, containing the cytoplasmic secretion ATPase GspE and the membrane proteins GspL, GspM, GspC and GspF.

The pseudopilus is a key element of the type 2 secretion system (T2SS) that forms a helical fiber spanning the periplasm. The fiber is assembled from multiple subunits of the major pseudopilin GspG(4,5,10-14). The pseudopilus is thought to form a plug of the secretin pore in the outer membrane and/or to function as a piston during protein secretion. In recent years, studies of the T2SS pseudopilins led to structure determinations of all individual pseudopilins (13,15-17). The recent structure of the helical ternary complex of GspK-GspI-GspJ suggested that these three minor pseudopilins form the tip of the pseudopilus(17). A crystal structure of GspG from Klebsiella oxytoca was in a previous study combined with electron microscopy data to arrive at a helical arrangement, with no evidence for special features, such as disulphide bridges, other covalent links or metal binding sites, for stabilizing this major pseudopilin or the pseudopilus(13).

The pseudopilins of the T2SS share a common fold with the type 4 pilins (15-21). Pilins are proteins incorporated into pili, long appendages on the surface of bacteria forming thin, strong fibers with multiple functions (19,21). Pilins and pseudopilins contain a pre-pilin leader sequence which is cleaved off by a pre-pilin leader peptidase.
A distinct feature of the type 4 pilins is the occurrence of a disulfide bridge connecting β4 to a Cys in the so-called “D-region” near the C-terminus (21). In a recent paper (23) on the thin fibers of Gram-positive bacteria, isopeptide units appeared to be essential for providing these filaments sufficient cohesion and stability. A key question was therefore if the major pseudopilin GspG also requires a special feature to obtain sufficient stability to perform its function.

EXPERIMENTAL PROCEDURES

Expression, purification and crystallization of V. cholerae GspG—The gene fragment corresponding to the soluble domain of V. cholerae GspG (residues 26-137) was cloned into a pCDFDuet-1 based vector (Novagen) for expression with an N-terminal hexahistidine tag followed by a TEV cleavage site. BL21(DE3) E. coli cells were grown in LB medium at 37°C, and induced for 3 hrs with 0.5 mM IPTG at 30 °C. GspG was purified from the soluble fraction of the lysed cells using Ni-NTA (Qiagen) followed by His-tag cleavage with TEV, an ion-exchange purification step using a 30Q column (GE Healthcare) and a final size-exclusion on a Superdex75 column (GE Healthcare) for both ion exchange peaks. After dialysis against 10 mM Na acetate, pH 5.0, crystals were obtained only for the first ion-exchange peak. The optimized crystals were grown using the vapor diffusion method in 22.5% PEG 3350, 0.1 M Na acetate pH 5.0, 0.04 M Zn acetate.

Structure determination of V. cholerae GspG—The crystals diffracted to ~4 Å initially. After 2 cycles of crystal annealing by blocking the cryo stream the resolution limit improved to 2.5 Å and a preliminary dataset was collected in house (Supplementary Table 1). Data were processed using HKL2000(24). The structure was solved by molecular replacement with Phaser(25) using the structure of K. oxytoca GspG(13) as a model. In the search model, the non-equivalent residues were truncated to Ala/Gly and the swapped β4-strand was removed. After density modification with Resolve(26), it was apparent that there are several metal ions in the structure. Those ions were initially thought to be Zn²⁺ ions present in the crystallization solution. Therefore, the data were collected at Zn remote and inflection wavelengths at beamline BL9-2 at SSRL.

Using anomalous data from either Zn remote or a combination of Zn remote/inflection datasets, SHELXD(27) found 3 Zn positions, 2 with full occupancy and 1 with ~50% occupancy. The initial model was improved using ARP/wARP(28), and finalized using manual rebuilding in Coot (29) alternating with refinement by REFMAC5(30) using 1 TLS group per chain. From subsequent tests it appeared that the structure could have been solved ab initio using the Zn anomalous signal. The two monomers in the asymmetric unit (ASU) form an anti-parallel dimer with 2 Zn²⁺ ions in the interface and a third Zn²⁺ ion participating in crystal contact with a symmetry-related molecule (Fig. 1). The two monomers superimpose with r.m.s.d. of 0.4 Å over 112 Ca atoms.

Expression and purification of V. vulnificus and EHEC GspG—The gene fragments corresponding to the soluble domain of V. vulnificus GspG (residues 26-137) and EHEC GspG (residues 17-136) were cloned into a pCDFDuet-1 based vector similar to V. cholerae GspG. The expression was performed as for V. cholerae GspG but LB media was supplemented with 1 mM calcium chloride, but no calcium chloride was added during subsequent purification and crystallization. The purification followed the procedure outlined for V. cholerae GspG with the first ion-exchange peak used for crystallization.

Crystallization and structure determination of V. vulnificus GspG—Crystals of V. vulnificus GspG were grown using vapor diffusion method in 1.95 M DL-malic acid pH 5.0. A dataset was collected at beamline BL9-2 at SSRL (Supplementary Table 1). Data were processed using XDS(31). The structure was solved by molecular replacement with Phaser(25) using the V. cholerae GspG structure as a model. After manual rebuilding in Coot(29), the three monomers in the ASU were refined with REFMAC5(30) using 3 TLS group per chain as defined by the TLSMD server(32). The three subunits in the ASU superimpose pairwise with an r.m.s.d. of 0.2-0.4 Å over 111 Ca atoms. The V. vulnificus GspG structures superimpose onto the V. cholerae GspG structures with an r.m.s.d. of 0.6-0.8 Å over 111 Ca atoms and 91% sequence identity in the superimposed region.
Crystallization and structure determination of EHEC GspG—A cluster of crystals was obtained during screening in a condition containing 1.0 M Na citrate, 0.1 M CHES pH 9.5. An individual crystal was separated from cluster, frozen and used for data collection. Data were collected at beamline BL9-2 at SSRL and processed using XDS(31). The structure was solved by molecular replacement with Phaser(25) using the V. cholerae GspG structure, with the Ca²⁺-binding loop and C-terminal α-helix removed, as a search model. The two monomers in the ASU were rebuilt using ARP/wARP(28) and finalized using Coot(29). The structure was refined with REFMAC5(30) using 3 TLS groups per chain as defined by the TLSMD server(32). The two subunits in the ASU superimpose with an r.m.s.d. of 2.0 Å over 114 Cα atoms. Large differences occur only in the N-terminal region where helix α1 unwinds. The exclusion of residues 18-29 from the superimposition reduces the r.m.s.d. to 0.5 Å. Superimposition onto the V. cholerae GspG structure gives an r.m.s.d. of 1.9-2.1 Å over 105 Cα atoms with 65% sequence identity in the superimposed region.

Functional secretion studies of GspG mutants—The ΔgspG strain and the complementing pMMB67EH-gspG plasmid were constructed as previously described(33). Mutations were introduced in gspG gene with QuickChange II Site-Directed Mutagensis Kit (Stratagene) using pMMB-gspG as a template. Primers used for the site change in gspGD116A and gspGD127A were 5'-gttcaccttagtgccggcggtcaagaaggtggtg-3, 3'-caagtggatcaagccggccagttctccacac-5 and 5'-gaaggtacccggtgccgctatcggtaactgg-3, 3'-cttccatggccacggcgatagccattgacc-5, respectively. gspGD116AD127A was then constructed using pMMB-gspGD116A as a template and the above primers specific for the gspGD127A site change. All mutations were verified by sequencing.

Detection of secreted protease activity.—Cultures were grown overnight in Luria broth (LB) supplemented with 100 μg/ml thymine and 200 μg/ml carbenicillin and centrifuged to separate the supernatant and cellular material. The supernatants were centrifuged once more and the protease activity was measured as described previously(4).

Immunoblotting for GspG.—Cultures were grown to mid-log phase. Whole cell lysates were prepared by resuspending cells in SDS-PAGE sample buffer with 50mM DTT and boiled for 5 minutes. 10 μl of OD₆₀₀=1.0 was loaded onto NuPage 4-12% Bis-Tris gradient gels (Invitrogen). Following electrophoresis and immunoblotting GspG was detected with rabbit polyclonal antibodies against V. cholerae GspG, kindly supplied by Dr. Michael Bagdasarian, Michigan State University, horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) and ECL Plus Western Blotting detection reagent (Amersham/GE Healthcare). Typhoon Trio variable mode imager system and ImageQuant software were used for imaging.

Fluorescence-based thermal shift assay—The soluble domain of V. cholerae double D116A/D127A mutant GspG gene (residues 26-137) was expressed and purified similar to the wild type protein. A single peak was obtained after the ion-exchange step. The thermal stability of proteins was measured by fluorescence using Sypro Orange as fluorophore as described elsewhere(34), either without additives, or in the presence of 1 mM calcium chloride, or in the presence of 1 mM EDTA. Four independent values were measured for each condition. The melting temperature (Tm) of wild type GspG was 50.0±1.0, 56.2±0.3 and 40.1±0.4 °C for no additive, calcium chloride and EDTA conditions, respectively. Tm values for the double D116A/D127A mutant were 46.4±0.6, 46.4±0.5 and 46.2±0.4 °C for no additive, calcium chloride and EDTA conditions, respectively.

Structure analysis—Multiple sequence alignments were made using ClustalW2(35). ESPript(36) was applied to render the alignments. The figures were generated using PyMol(37).

RESULTS AND DISCUSSION

We solved the crystal structures of V. cholerae, V. vulnificus and EHEC GspG’s, all to better than 2.0 Å resolution, yielding a total of seven views of these three GspG molecules (Supplementary Table 1, Figs. 1 and 2, Supplementary Fig. 2). All these structures largely agree with the structure of K. oxytoca GspG and show the typical pilin fold composed of an N-terminal α-helix, a "variable segment" and a C-
terminal β-sheet. However, we consistently found two additional structural features that have not been described previously. First, the C-terminal residues in all new GspG structures adopt a helical conformation (Figs. 1 and 2, Supplementary Fig. 2) in contrast with a β-strand observed in K. oxytoca GspG. In the latter structure, a β-strand swap occurred between the two pseudopilin molecules in the asymmetric unit(13). Second, a distinct high electron density is present in each of the two subunits of the V. cholerae GspG structure, surrounded by the carboxylates of Asp116 and Asp127 and by four main chain carbonyl oxygens (from Leu113, Gln118, Gly120 and Gly125) in an octahedral manner.

The high density of the peak (Fig. 1B) and the all-oxygen sphere of ligands provided initial evidence that the ion bound is a calcium ion. Additional evidence that the two ions located in the β2- β3 loop are calcium ions is: (i) the anomalous difference maps using the λ = 0.97946 Å data reveal 4.7 and 3.6 sigma peaks (Fig. 1B) at the Ca²⁺-binding sites which is proportional to the data reveal 4.7 and 3.6 sigma peaks (Fig. 1B) at the Ca²⁺-binding sites using the anomalous difference maps (Supplementary Fig. 1). Nevertheless, in the EHEC GspG structure both subunits show a high electron density consistent with Ca²⁺-binding in essentially the same position as in V. cholerae and V. vulnificus GspG’s. The latter proteins have an insert of 3 residues, forming helix α3 in the Vibrio GspG structures, between the two conserved calcium-coordinating aspartates (Fig. 1C, Supplementary Fig. 1). Nevertheless, in the EHEC GspG structure both subunits show a high electron density consistent with Ca²⁺-binding in essentially the same position as in V. cholerae and V. vulnificus GspG. The temperature factors for the metal ions with full occupancy are 34.2 Å² in chain A and 27.6 Å² in chain B which are close to the average temperature factors of 21.2 Å² and 18.7 Å², respectively, for the coordinating atoms. The charged ligands of the EHEC calcium site are Asp117 and Asp125, which are structurally equivalent to Asp116 and Asp127 of V. cholerae GspG, with as new ligand the hydroxyl of Thr122, and with the carbonyl oxygens of Pro114 and Val119 completing the plane of a trigonal bipyramidal coordination (Fig. 2B). The all oxygen coordination of the ion site is in agreement with Ca²⁺-binding.

The amino acid sequences of GspG’s from EHEC and K. oxytoca align well mutually but display distinct sequence variation in the Ca²⁺-binding region compared to that of V. cholerae GspG (Supplementary Fig. 1). Nevertheless, in the EHEC GspG structure both subunits show a high electron density consistent with Ca²⁺-binding in essentially the same position as in V. cholerae and V. vulnificus GspG. The temperature factors for the metal ions with full occupancy are 34.2 Å² in chain A and 27.6 Å² in chain B which are close to the average temperature factors of 21.2 Å² and 18.7 Å², respectively, for the coordinating atoms. The charged ligands of the EHEC calcium site are Asp117 and Asp125, which are structurally equivalent to Asp116 and Asp127 of V. cholerae GspG, with as new ligand the hydroxyl of Thr122, and with the carbonyl oxygens of Pro114 and Val119 completing the plane of a trigonal bipyramidal coordination (Fig. 2B). The all oxygen coordination of the ion site is in agreement with Ca²⁺-binding.

The GspG family sequence alignment (Supplementary Fig. 1) indicates that the two Ca²⁺-coordinating aspartates are conserved, are rarely a Glu, and only in Legionella pneumophila one is an alanine (not shown). Note also how the Thr/Ser presence in the Ca²⁺-binding site correlates consistently with the omission of three residues, forming the V. cholerae GspG helix α3, in the β2-β3 loop. There appear therefore to be two subclasses of major pseudopilins. One, a Vibrio-like subclass, with four main chain carbonyl oxygen calcium binding ligands plus two carboxylate ligands, and a short helix α3 between the two carboxylate-providing amino acids. A
second, EHEC-like subclass, with two main chain calcium binding ligands, one Thr/Ser side chain oxygen ligand plus two carboxylate ligands, and no helix between the two carboxylate-providing amino acids. It is most likely that K. oxytoca GspG belongs to the latter subclass and that the 8-strand swap in the crystal structure(13) disrupted the calcium-binding site.

The functional importance of Ca\(^{2+}\)-coordination by GspG was assessed by replacing the highly conserved residues Asp116 and Asp127 in V. cholerae GspG with alanines and monitoring the effect on extracellular secretion of protease by V. cholerae. No protease secretion was observed when plasmid-encoded GspG- D116A/D127A was expressed in a mutant strain lacking GspG (Fig. 3), indicating that the simultaneous removal of the two aspartate carboxylates prevents T2SS function by impairing Ca\(^{2+}\)-binding. The singly substituted variants, GspG-D116A and GspG-D127A, remained functional (Fig. 3A), presumably because either carboxylate in conjunction with the main chain carbonyls from Leu113, Gln118, Gly120, Gly125 is still capable of Ca\(^{2+}\)-binding. Immunoblot analysis of cell extracts from the ΔgspG mutant strain showed that the single and the double mutant variant proteins were expressed at levels similar to that of wild type GspG (Fig. 2A). We also measured thermal stability of the wild-type and the double D116A/D127A mutant GspG using a fluorescence-based thermal shift assay(34,38,39) and found that the mutant is slightly less stable than wt GspG, with a T\(_m\) of 46.4 °C vs. 50.0 °C. Interestingly, the stability of the double mutant was unaffected by adding Ca\(^{2+}\) or EDTA, whereas the stability of the wild type protein increased substantially upon adding Ca\(^{2+}\) (T\(_m\) 56.2 °C) and decreased upon adding EDTA (T\(_m\) 40.1 °C).

These studies clearly indicate that altering the coordinating aspartates of the Ca\(^{2+}\)-site in V. cholerae GspG dramatically impairs the functioning of the T2SS. The loss of calcium binding by GspG could influence protein secretion by the T2SS in a number of ways, for instance by possibly promoting a conformation of the C-terminal helix conducive for favorable interactions in the pseudopilus (Supplementary Fig. 3).

It appears that three major fibrous arrangements used by bacterial secretion systems are dependent on entirely different stabilizing features: disulfide bridges in the type 4 pilins (21), isopeptide bonds in the thin fibers of Gram-positive bacteria(23), and calcium ions in the pseudopilus of the T2SS (Figs. 1 and 2). Intriguingly, an unrelated dinuclear calcium site is observed and conserved in the “tip” pseudopilin GspK(17). Hence, the T2SS is dependent on calcium-binding in multiple ways.

REFERENCES


**FOOTNOTES**

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**Accession codes.** Protein Data Bank: Coordinates and structure factors have been deposited with accession codes 3FU1 for *V. cholerae* GspG, 3GN9 for *V. vulnificus* GspG, and 3G20 for EHEC GspG.
FIGURE LEGENDS

Fig. 1. Structure and calcium binding site of *V. cholerae* GspG. *A*. The two *V. cholerae* GspG subunits per asymmetric unit (AU) are shown in yellow and purple. Orange and grey spheres indicate calcium and zinc ions respectively. Side chains of residues coordinating calcium or zinc ions are shown in stick representation. *B*. Stereo representation of electron density in the Ca$^{2+}$-binding site. The 2Fo-Fc map is contoured at 1.2 sigma displayed as grey mesh. The calcium ion is surrounded by anomalous difference Fourier electron density colored in orange, calculated using data to 2.5 Å resolution collected at $\lambda=1.28344\AA$, and contoured at 4 sigma. For the essentially identical arrangement in *V. vulnificus* GspG, see Supplementary Fig. 2. *C*. Sequence alignment of the Ca$^{2+}$-binding regions of the four major pseudopilins with known structure (*V. cholerae*, *V. vulnificus*, EHEC and *K. oxytoca* GspG). Residues involved in Ca$^{2+}$-binding, either by main chain or side chain oxygens, are highlighted in orange. The conserved Ca$^{2+}$-site aspartate ligands are indicated with an orange asterisk on top and below; the liganding Thr in EHEC, and by analogy Ser122 in *K. oxytoca* GspG, with an orange asterisk below. Secondary structure elements above and below displayed sequences correspond to *V. cholerae* and EHEC GspG structures, respectively.

Fig. 2. Structure and calcium-binding site of EHEC GspG. *A*. The two EHEC GspG subunits per asymmetric unit are shown in light and dark blue. Orange spheres indicate calcium ions. Side chains of residues coordinating calcium ions are shown in stick representation, as are buffer CHES molecules. *B*. Stereo representation of electron density in the Ca-binding site. 2Fo-Fc map contoured at 1.2 sigma displayed as grey mesh.

Fig. 3. Simultaneous substitution of Asp116 and Asp127 results in *V. cholerae* GspG inactivation. *A*. Wild type and *gspG* mutant strains of *V. cholerae* containing either pMMB67 or pMMB-gspG variants were grown in LB. Culture supernatants were tested for the presence of extracellular protease. The rate of hydrolysis was obtained from three independent experiments and the results are presented with standard error (+/- SEM). *B*. Cells were disrupted and subjected to SDS-PAGE and immunoblotting with anti-GspG antibodies to determine the relative level of *V. cholerae* GspG expression. The positions of molecular mass markers are shown on the left and *V. cholerae* GspG variants on the right.
Figure 2

A

B
Figure 3

A

![Graph showing FUrmin/OD600 values for different conditions.]

B

![Image of a gel showing bands for GspG.]
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