Journal of Structural Biology xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Structural insights into the architecture of the *Shigella flexneri* virulence factor IcsA/VirG and motifs involved in polar distribution and secretion

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ARTICLE INFO

Article history:
Received 26 October 2016
Received in revised form 23 January 2017
Accepted 3 March 2017
Available online xxxx

Keywords:
VirG
IcsA
Virulence factor
Autotransporter

ABSTRACT

IcsA/VirG is a key virulence factor of the human pathogen *Shigella flexneri*, acting as both an adhesin and actin-polymerizing factor during infection. We identified a soluble expression construct of the IcsA/VirG α-domain using the ESPRIT library screening system and determined its structure to 1.9 Å resolution. In addition to the previously characterized autochaperone domain, our structure reveals a new domain, which shares a common fold with the autochaperone domains of various autotransporters. We further provide insight into the previously structurally uncharacterized β-helix domain that harbors the polar targeting motif and passenger-associated transport repeat. This structure is the first of any member of the recently identified passenger-associated transport repeat-containing autotransporters. Thus, it provides new insights into the overall architecture of this class of autotransporters, the function of the identified additional autochaperone domain and the structural properties of motifs involved in polar targeting and secretion of the *Shigella flexneri* virulence factor IcsA/VirG.

1. Introduction

Shigella flexneri is a Gram-negative, highly adapted human-pathogenic bacterium and the causative agent of shigellosis/bacillary dysentery (Levine et al., 2007). This foodborne disease manifests itself in severe bloody diarrhea and occurs primarily in developing countries where it is endemic (Bennish and Wojtyniak, 1991) with more than 160 million cases and 1.1 million deaths per year worldwide, mainly caused by *S. flexneri* (Kotloff et al., 1999). In addition, shigellosis is the primary cause of traveler's diarrhea in individuals visiting developing countries. Treatment and prevention of *Shigella* infections is hindered by the large number of multidrug-resistant strains and the lack of an efficient vaccine. *Shigella* utilizes a variety of virulence-plasmid encoded factors for the efficient infection *via* the fecal-oral route; these facilitate intracellular survival in the cytosol of epithelial cells of the small intestine and subsequent spreading into neighboring tissue. Structural and functional characterization of these key virulence factors and a detailed understanding of molecular mechanisms underlying *Shigella* pathogenesis will thus pave the way for new treatments and vaccines against shigellosis.

The *icsA* (intra-cellular spread gene A) gene, also termed *virG* (virulence gene G) is located on the *S. flexneri* virulence plasmid (Venkatesan et al., 2001). It encodes the *S. flexneri* outer membrane protein and virulence factor IcsA/VirG (hereafter referred to as VirG), with two major functions; as recently shown, during the early stage of the infection cycle, *Shigella* cells reside in the intestinal lumen where VirG acts as an adhesin binding to a still unknown receptor on the host cell surface (Brotcke Zumsteg et al., 2014). After internalization, VirG exerts its second function in the host cell cytoplasm by mediating intracellular actin-based motility (ABM) *via* the activation of N-WASP (neural Wiskott-Aldrich syndrome protein). VirG is the only *Shigella* protein that is essential for ABM, as VirG-expressing *E. coli* cells also show ABM *in vitro* (Goldberg and Theriot, 1995). Interestingly, VirG shows different proteolytic digestion patterns, depending on which of the two functions it is exerting, indicating that the two states adopt different conformations (Brotcke Zumsteg et al., 2014). The key role of VirG in pathogenicity becomes obvious upon its deletion, resulting in loss of bacterial intracellular actin assembly, loss of cell-to-cell spread combined with a markedly reduced virulence in humans and animal models (Bernardini et al., 1989; Coster et al., 1999; Lett et al., 1989; Makino et al., 1986; Sansonetti et al., 1991).

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http://dx.doi.org/10.1016/j.jsb.2017.03.003
1047-8477/

VirG is composed of three major domains (Fig. 1A); residues 1–52 cover an atypical signal peptide (Brandon et al., 2003), responsible for the secretion into the periplasm. The passenger- or α-domain, including residues 53–758, is presented on the cell surface. The β–domain, comprising amino acids (aa) 759–1102, adopts a β-barrel fold and anchors VirG to the bacterial outer membrane (Suzuki et al., 1995). VirG is a member of the type Va secretion system (Henderson et al., 2004), also called an autotransporter (AT) protein or, more precisely, a self-associating autotransporter (SAAT) as this class of AT homo-oligomerizes (Grabowicz, 2010; Teh and Morona, 2013). Already in the cytoplasm, VirG localizes to the old pole of the bacterial cell, where it is secreted by the sec-pathway into the periplasm (Jain et al., 2006; Janakiraman et al., 2009). Subsequently, the signal peptide is removed by a signal peptidase (Brandon et al., 2003) and the β-domain inserts into the outer membrane. The passenger domain is exported by the β-domain to be presented on the surface of the bacterium (Suzuki et al., 1995). The distribution of VirG observed in the cytoplasm is retained on the bacterial surface, where it forms a gradient with the highest concentration at the old cellular pole. The gradient is further reshaped by the activity of the specific protease IcsP, which cleaves VirG between positions 758 and 759, resulting in the release of the α-domain (Egile et al., 1997). The presence of lipopolysaccharide (LPS) is essential for the correct localization of VirG, as defects in LPS result in abnormal distribution of VirG on the cellular surface (Rajakumar et al., 1994; Sandlin et al., 1995).

The surface exposed α-domain contains a number of subdomains and regions with annotated functions. The region containing residues 117–332 is formed by a series of glycine-rich repeats (GRRs) (Goldberg et al., 1993). Residues 103–433, which comprise the GRRs and additional N- and C-terminal stretches, are sufficient for the interaction with the actin polymerization regulator N-WASP *in vitro* (Suzuki et al., 1998). For N-WASP interaction *in vivo* and actin tail formation however, residues 53–729 are required, i.e. essentially the whole α-domain (Teh and Morona, 2013). Activated N-WASP in turn activates the Arp2/3 complex, which generates a propulsive force by forming branched actin tails, typical for the intracellular motility of *Shigella* cells (Egile et al., 1999; Moreau et al., 2000). Only recently, residues 507–758 were shown to interact with apyrase, a periplasmic *Shigella* enzyme, which has been shown to be important for the unipolar localization of VirG and intercellular spread (Wang et al., 2014). A region encompassing residues 320–433 binds both the human autophagy protein ATG5 and *Shigella* IcsB and plays a role in the targeting of *Shigella* cells for degradation by autophagy (Ogawa et al., 2005). Residues 634–735 form the autochaperone domain, which assists in the folding of the α-domain by forming a structured nucleus, once it is passed by the β-domain (Oliver et al., 2003; Peterson et al., 2010; Soprova et al., 2010). A stable fragment of this domain comprising residues 591–740 has been identified by limited proteolysis and its structure was solved by X-ray crystallography (Kuhnel and Diezmann, 2011). The stretch between residues 532–570 is called the polar targeting (PT) region, because it is sufficient to localize VirG to the old pole of the bacterial cell (Doyle et al., 2015a), resulting in an asymmetrical distribution of actin-polymerization and thus permitting the directed actin-based propulsion required for efficient inter- and intracellular spreading. Interestingly, a conserved passenger-associated transport repeat (PATR), involved in passenger secretion efficiency, has recently been identified in VirG spanning amino acids 526–557, thereby overlapping with the polar targeting region (Doyle et al., 2015b).

Apart from the autochaperone domain, no structural data exist for VirG, largely due to the lack of soluble and stable recombinant VirG protein. To address open questions regarding overall domain architecture as well as the position and structural properties of the PATR and PT region, we first identified soluble VirG expression constructs using a library-based construct screening technology (ESPRIT: expression of soluble protein by random incremental truncation), then performed structural characterization using X-ray crystallography.

2. Materials and methods

2.1. Identification of soluble VirG constructs using ESPRIT

The ESPRIT system permits identification of well-behaving constructs of poorly understood proteins through generation of randomly truncated gene inserts coupled to a high throughput colony-based solubility screen (Yumerefendi et al., 2010). Detailed protocols for other genes have been described (Angelini et al., 2009; Tarendeau et al., 2007). Briefly, DNA encoding VirG residues 1–758 and 1–592 was subcloned into pESPRIT002, a pET9a-derived plasmid that fuses an in-frame C-terminal biotin acceptor peptide (BAP), plus an N-terminal TEV-cleavable hexahistidine tag sequence with restriction sites for unidirectional truncation of the gene insert with exonuclease III and mung bean nuclease. Truncation reactions were performed and plasmids recovered by ligation and transformation. One-third of plasmids therefore encoded a hexahistidine tag fused in-frame to all possible N-terminal residues of VirG-BAP. *E. coli* BL21 AI (RIL) was transformed with plasmid libraries and colonies titrated on LB agar with antibiotics, then picked robotically into 384-well plates. In total, 18,432 VirG clones were isolated representing an approximate three-fold oversample of each truncated variant of both starting constructs.

For expression screening, inocula were arrayed robotically at high density onto nitrocellulose membranes over LB agar with antibiotics at 37 °C as described (Angelini et al., 2009) and incubated until colonies became visible. Membranes were moved onto fresh agar containing antibiotics and arabinose inducer at 25 °C, 4 h, then colonies lysed with NaOH. Membranes were hybridized with streptavidin-Alexa 448 fluorophore (Life Technologies) and a monoclonal anti-hexahistidine tag antibody with associated Alexa532 secondary antibody. Colony filters were imaged with a fluorescence scanner and 96 clones exhibiting strongest hexahistidine tag and BAP signals were isolated from the library. Small scale test-expressions were performed and soluble protein levels were detected by western-blotting and by Coomassie-stained SDS gels. The clones were sequenced and interesting constructs taken into large scale protein production.

2.2. VirG-constructs protein preparation

Between 8 l and 16 l of TB were inoculated from an overnight pre-culture to OD 0.03 and grown at 37 °C, 130 rpm to OD 0.4. The temperature was reduced to 20 °C and the cultures were further grown to OD 0.6–0.8 where they were induced by the addition of 0.2% (w/v) arabinose. Subsequently the cultures were further incubated overnight. Cells were pelleted at 4 °C, 5000 rpm for 10 min, the pellets were resuspended in lysis buffer (50 mM HEPES pH 7.0, 300 mM NaCl, 5 mM β-mercaptoethanol (BME)) supplemented with 1 mM phenylmethylsulfonylfluoride (PMSF), 5 mM $MgCl_2$, 400 μg DNase, 40 mg lysozyme and cells were lysed twice using a homogenizer. The lysate was cleared by centrifugation at 4 °C, 16,000 rpm for 45 min. Ni-NTA resin (Qiagen) and 20 mM imidazole were added to the cleared lysate that was then stirred at 4 °C for 1 h for protein binding. Beads were loaded into a gravity flow column and washed using 50 column volumes (CV) of washing buffer (50 mM HEPES pH 7.0, 300 mM NaCl, 5 mM BME, 30 mM imidazole). Protein was eluted with 10 CV of elution buffer (50 mM HEPES pH 7.0, 300 mM NaCl, 5 mM BME, 250 mM imidazole). TEV

Please cite this article in press as: Leupold, S., et al. Structural insights into the architecture of the *Shigella flexneri* virulence factor IcsA/VirG and motifs involved in polar distribution and secretion. J. Struct. Biol. (2017), http://dx.doi.org/10.1016/j.jsb.2017.03.003