Selective binding of virulence type III export chaperones by FliJ escort orthologues InvI and YscO

Lewis D.B. Evans & Colin Hughes

Department of Pathology, Cambridge University, Cambridge, UK

Correspondence: Lewis D.B. Evans, Department of Pathology, Cambridge University, Tennis Court Road, Cambridge CB2 1QH, UK. Tel.: +44 1223 3333 733; fax: +44 1223 336926; e-mail: le227@cam.ac.uk

Received 16 September 2008; accepted 2 February 2009. First published online 2 March 2009.

DOI:10.1111/j.1574-6968.2009.01535.x

Editor: Mark Schembri

Keywords
type III export; secretion pilots; chaperone escort.

Abstract

Bacteria secrete flagella subunits and deliver virulence effectors via type III export systems. During flagellar filament assembly, a chaperone escort mechanism has been proposed to enhance the export of early, minor flagellar filament components by selectively binding and cycling their chaperones. Here we identify virulence orthologues of the flagellar chaperone escort FliJ and show that the orthologues Salmonella InvI and Yersinia YscO are, like FliJ, essential for their type III export pathway and similarly, do not bind export substrates. Like FliJ, they recognize a subset of export chaperones, in particular those of the host membrane translocon components required for subsequent effector delivery.

Introduction

Type III secretion systems enable Gram-negative bacteria to assemble cell surface flagella (Aizawa, 2001) and deliver virulence effectors to eukaryotic cells (Galan & Wolf-Watz, 2006). The integral membrane components of the export machineries are closely related (Kubori et al., 1998), and in both cases cytosolic chaperones pilot cognate late export substrates to dock at the membrane-associated ATPase complex (Auvray et al., 2002; Gauthier & Finlay, 2003; Thomas et al., 2004; Akeda & Galan, 2005). The export processes are ordered: the flagellar basal body/rod/hook substructures are assembled before filament subunits are exported, while completion of the virulence needle complex initiates export and assembly of the translocon into the eukaryotic cell membrane to allow delivery of effectors (Homma et al., 1984; Sukhan et al., 2001). This order is governed in part by a substrate specificity switch from rod/hook to filament subunits or from needle to translocon components and effectors (Fraser et al., 2003; Sorg et al., 2007), during which early components and late export substrates are suggested to be sorted after docking at each export machinery (Stafford et al., 2007; Riordan & Schneewind, 2008). We have proposed that an additional mechanism could operate during the late export of flagellar filament subunits, as the minor filament substructures (the hook–filament junction and filament cap) must assemble before the large number of major filament (flagellin) subunits can be incorporated (Homma et al., 1984). We have reported that a novel escort protein FliJ located in the export ATPase complex at the base of the export apparatus can recruit and cycle-free unloaded chaperones of minor substructure subunits. As FliJ does not recognize the chaperone of the major subunit flagellin (Evans et al., 2006), we proposed that it could preferentially enhance the formation of minor chaperone–subunit complexes and thus favour assembly of the filament junction and cap. This would be beneficial as these minor subunits are thought to compete for export with the major subunit (Homma et al., 1984; Kubori et al., 1998).

Here, we assess whether a similar FliJ-like chaperone escort activity could operate late in the virulence type III pathway, i.e. after needle complex assembly. We assessed whether the putative FliJ orthologues Salmonella typhimurium InvI and Yersinia enterocolitica YscO could similarly selectively recognize free chaperones that facilitate export of late substrates destined for the host cell.

Materials and methods

Bacterial strains and plasmids

Bacteria were cultured at 37°C to the late exponential phase (A600nm 2.0), unless stated, in Luria–Bertani broth.
containing, where appropriate, ampicillin, chloramphenicol or kanamycin (at 50–20 μg mL⁻¹). In vivo studies were performed in wild-type S. typhimurium SJW103, and chromosomal deletion mutant invE::Km, in which the gene was replaced by a kanamycin resistance cassette, was constructed using the Red recombinease system (Datsonk & Wanner, 2000). Recombinant proteins were expressed in Escherichia coli C41 from isopropyl-β-D-thiogalactoside-inducible plasmids.

Recombinant plasmids encoding individual virulence chaperones, export substrates and putative FliJ orthologues genes were constructed by PCR amplification using Pfu turbo DNA polymerase from S. typhimurium genomic DNA and Y. enterocolitica virulence plasmid pYVe227. To make glutathione-S-transferase (GST) fusions of InvI and YscO, genes were amplified by PCR, and products were inserted BamHI/XhoI into pGEX-4T-3 (GE Healthcare). PCR products of Salmonella sicA, invB, sigE and sicP and Yersinia sycD, sycE, sycH, sycO, sycT, yopD, yopE, yopH, yopO and yopT were inserted either NdeI/BamHI or NdeI/HindIII into pACT7 (Kaelin et al., 1992) or pET15b (Novagen). Histidine-tagged recombinant plasmids of Salmonella virulence genes (sipA, sipB, sipC, sipD, sopE and sipP) were gifted by the Koronakis laboratory. Inserts were verified by DNA sequencing. Recombinant genes encoding InvI (and N/C terminally histidine-tagged InvI) were constructed by PCR and inserted XbaI/HindIII into pBAD18 (Guzman et al., 1995).

Purification of proteins

Detailed purification protocols have been published previously (Hayward & Koronakis, 1999; Hayward et al., 2000; McGhie et al., 2001). In brief, cells expressing individual histidine-tagged recombinant proteins were resuspended in buffer A containing phosphate buffer, NaCl and detergent [50 mM NaH₂PO₄ (pH 7.4–8.6), 150–300 mM NaCl, 1 mM dithiothreitol and 0–0.5% Triton X-100 (v/v)], before lysis in a French pressure cell (Amino). Cleared cell lysates were passed over nickel nitritoltriacetic acid (N²⁺) agarose (Qia-gen) and recombinant proteins were eluted using imidazole. Recombinant proteins SipB, SipC, YopD, YopT and YopE were purified under denaturing conditions (6 M guanidinium chloride) from the insoluble fractions and elution fractions were dialysed in series first, against buffer A containing 0.5 M pyridinio propanesulphonate, followed by buffer A alone.

Affinity chromatography copurification assays

Copurification of protein complexes was achieved with either N²⁺ agarose or glutathione sepharose 4B as described previously (Evans et al., 2006). Chaperone prey proteins were native, whereas purified effector prey proteins (SipA, SipB, SipC, SipD, SopE, SptP, YopD, YopE, YopH, YopO and YopT) were histidine tagged. In vitro mixed purified proteins or cleared cell lysates were incubated for 2 h with affinity resin. After extensive washing [buffer A (10–60 mM imidazole)], proteins were eluted in sodium dodecyl sulfate (SDS) loading buffer. For in vivo studies, soluble lysates of S. typhimurium strains expressing His-InvI at an export complementing level from arabinose (0.01%)-inducible plasmid pBAD18 (Guzman et al., 1995) were prepared as above, incubated for 1 h with N²⁺ agarose, washed three times with buffer A (60 mM imidazole) and proteins eluted in SDS loading buffer; untagged InvI was used as a negative control.

Assay of virulence effector protein export

Salmonella typhimurium culture supernatants were clarified by centrifugation and passed through a 0.2-μm nitrocellulose filter (Millipore). Virulence proteins were precipitated by 10% (v/v) trichloroacetic acid on ice for 1 h, separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by immunoblotting with appropriate polyclonal antisera (Cain et al., 2004).

Results and discussion

While the bacterial type III export membrane components are generally obvious homologues, this is not so for the chaperones (Bennett & Hughes, 2000), which in the virulence systems bind effector N-terminal regions (Page & Parsot, 2002) rather than the flagella subunits C-terminal polymerization domains (Avrray et al., 2001). Nevertheless, virulence operons contain an essential (Collazo et al., 1995; Payne & Straley, 1998) but currently anonymous gene that, like fliJ, lies between the genes encoding the export ATPase and a protein known or suspected to control hook or needle length (Journet et al., 2003; Shibata et al., 2007) (Fig. 1). These virulence genes encode, in each case, a protein of a size similar to FliJ (14–18 kDa), and while these do not show significant sequence similarity to FliJ, they are predicted to have comparably high helicity (data not shown).

To establish whether S. typhimurium InvI and Y. enterocolitica YscO proteins could have a chaperone escort function analogous to FliJ, we applied in vitro affinity chromatography to identify possible recognition of free unloaded chaperones that facilitate export of late substrates destined for the host cell. A representative set of late virulence chaperones was incubated with (E) and without (−) GST-InvI (46 kDa) or Yersinia YscO (46.6 kDa) with glutathione sepharose. Figure 2a shows that Salmonella GST-InvI bound the chaperone SicA (19 kDa), but not chaperones InvB (15 kDa) or SigE (13 kDa); SicP was poorly expressed and excluded from the study. Similarly (Fig. 2b), Yersinia GST-YscO recognized chaperone SycD (19 kDa), and less prominently the chaperone SycT (15 kDa), but not...
chaperones SycE (15 kDa), SycH (16 kDa) or SycO (17 kDa). We also assessed the ability of these putative escort orthologues to bind cognate partners of the recognized chaperones, as previously FliJ was reported to have general chaperone activity thought to interact with subunits of the flagellum (Minamino et al., 2000). These interactions were not detected in assays where escort–chaperone interactions were elucidated (Evans et al., 2006). Figure 2 shows that neither of the purified cognate partners of SicA, SipB (62 kDa) or SipC (42 kDa) bound to GST-InvI. Yersinia GST-YscO (Fig. 2) was also unable to recognize the purified cognate-binding partners of either SycD (YopD, 33 kDa; YopB was poorly expressed and excluded from the study) or SycT (YopT, 36 kDa). None of the other effectors tested, SipA, SipD, SopE, SptP, YopE, YopH or YopO, bound their respective FliJ orthologues (Supporting Information, Fig. S1). This indicates that InvI and YscO are not general chaperones. Purified cognate substrates assayed still bound their chaperones (Fig. S1), and no chaperone, translocon component or effector bound unfused GST (G) or glutathione sepharose alone (–) (Fig. 2).

Fig. 1. Selected virulence type III secretion operons aligned using COLIBASE (http://xbase.bham.ac.uk/), TIGR (http://www.tigr.org/) and NCBI (http://www.ncbi.nlm.nih.gov/) to the Salmonella typhimurium LT2 fli-K flagellar operon. These contain the chaperone escort gene fliJ located between the ATPase (fliI) and hook length control (fliK) genes.

Salmonella

![Salmonella Operon Diagram](image)

Fig. 2. Affinity chromatography following incubation with (E) and without (–) GST-tagged FliJ orthologue (InvI or YscO) or with unfused GST (G). (a) Salmonella InvI or (b) Yersinia YscO with cell lysates (load L) from Escherichia coli CA1 expressing the respective Salmonella and Yersinia chaperones and substrates (indicated by arrows). Samples were separated by SDS-PAGE (10/15%) and stained with Coomassie blue.
separated by SDS-PAGE (15%) and immunoblotted.

either control InvI or histidine-tagged InvI as above. Samples were

phy of lysates (L) from

SicA chaperone by functional histidine-tagged InvI. Affinity chromatogra-

1995), and

yscO

attenuates

Salmonella

fliJ

the

(Payne & Straley, 1998). These data are also compatible with

arabinose. This agrees with data showing that an

FEMS Microbiol Lett

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(2009) 292–297 c

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The data show that, like the flagellar escort FliJ, InvI is

essential for chaperoned and unchaperoned export, and InvI

and YscO do not bind export substrates and recognize a

subset of export chaperones. We could not demonstrate the

competitive acquisition of escort-bound chaperones by
cognate substrates evident in the flagellar system (Evans

et al., 2006). Also, we cannot rule out the possibility of a

tripartite complex of escort–chaperone and effector. The

results nevertheless provide support for the possibility that

an FliJ-like escort mechanism may similarly allow selective
cycling of virulence chaperones. What might be the advan-
tage of this? Although the significance of the weak YscO
interaction with SycT is unclear [the SycT partner YopT is a
cysteine protease effector (Aepfelbacher et al., 2003)], both

InvI and YscO bind the chaperones unequivocally for the

translocon components. Chaperone SicA binds translocon component SipC (Tucker & Galan, 2000) and possibly SipB (Kaniga et al., 1995), while SycD chaperones the translocon components YopB and YopD (Neyt & Cornelis, 1999a, b). Recent reports propose the notion of ordered export of late substrates (postcompletion of the needle complex) in the virulence system (Sorg et al., 2007). The SipB/C and YopB/D translocons are believed to insert into the host cell membrane and are essential for delivery of effectors destined for the interior of the host cell (Neyt & Cornelis, 1999a, b; Page et al., 1999; McGhie et al., 2002).

Preferential export of these membrane translocon components could increase the efficiency of effector delivery. Our data are compatible with an FliJ-like escort function for InvI and YscO, selectively recruiting translocon chaperones to enhance delivery of their cognate-binding partners.

Acknowledgements

We thank V. Koronakis and Emma McGhie for DNA and antisera, and R. Hayward for critically reading the
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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Affinity chromatography of effectors with (+) and without (−) GST-InvI or GST-YscO as in Fig. 2.

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