The FliS chaperone selectively binds the disordered flagellin C-terminal D0 domain central to polymerisation

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Abstract

Assembly of each Salmonella typhimurium flagellum filament requires export and polymerisation of ca. 30 000 flagellin (FliC) subunits. This is facilitated by the cytosolic chaperone FliS, which binds to the 494 residue FliC and inhibits its polymerisation. Yeast two-hybrid assays, co-purification and affinity blotting showed that FliS binds specifically to the C-terminal 40 amino acid component of the disordered D0 domain central to polymerisation. Without FliS binding, the C-terminus is degraded. Our data provide further support for the view that FliS is a domain-specific bodyguard preventing premature monomer interaction.

Keywords: Flagella assembly; Type III protein export

1. Introduction

The bacterial flagellar propeller is a tubular filament growing to a length of 15 μm by polymerisation of ca. 30 000 flagellin (FliC) subunits at its distal end [1,2]. In Salmonella typhimurium filament assembly is facilitated by the substrate-specific chaperone FliS [3]. Mutants lacking FliS are specifically attenuated in FliC export [3], and produce only short filaments [4]. Dimeric FliS forms a soluble cytosolic complex with the ca. 51.5-kDa (494 amino acids) FliC monomer and inhibits in vitro polymerisation into filaments [3]. We have therefore suggested that FliS acts as a bodyguard to control polymerisation [3].

FliC is folded into four morphological domains, D0–D3 (Fig. 1), and polymerisation is specifically dependent upon the D0 domain, which comprises the extreme N- and C-terminal regions conserved throughout bacterial flagellins [2,5–7]. D0 is structurally disordered [2,8] and as a consequence was excluded from the FliC three-dimensional crystal structure (D1–D3, amino acid residues 56–450, Fig. 1B [9,10]). These terminal disordered regions fold and simultaneously bind head to tail to the termini of subunits already polymerised, in turn becoming stabilised in the filament [2]. FliS binds within the C-terminal third of FliC [3], which spans D2 and the C-terminal components of D1 and D0 (residues 310–494, Fig. 1B). This suggests that FliS may target the disordered C-terminal component of the D0 polymerisation domain, but it remains possible that, alternatively or additionally, it binds the ordered helical regions of D1/2. Here we define more closely the FliC target sequence to better establish the role of FliS.

2. Materials and methods

2.1. Bacteria and recombinant DNA manipulations

Bacteria were grown at 37°C in Luria–Bertani (LB) broth or on LB agar, supplemented with ampicillin (100 μg ml⁻¹) and chloramphenicol (12.5 μg ml⁻¹) as necessary. Routine DNA manipulation and electroporation were carried out with Escherichia coli recA1 XL1 Blue (Stratagene) [11] and E. coli BL21 (DE3) [12] was used for overexpression. Cloning into the GST fusion expression vector pGex4T-3 (Pharmacia), or the T7 expression vectors pET15b (Novagen) and pACT7 [13], involved PCR amplification from chromosomal DNA (1–10 ng) of the wild-type S. typhimurium SJW1103, using 0.25 mM of each dNTP, 50 pmol oligonucleotide pairs, and
2.5 U native Pfu DNA polymerase (Stratagene). FliC internal deletions were generated by PCR amplification, using the splicing by overlap extension technique [14], in each case employing four oligonucleotides. Amplified DNA was purified using Qiaquick PCR purification kit (Qiagen), digested at enzyme sites encoded in the oligonucleotide 5′-ends, and ligated to the corresponding sites in the vector DNA. Mutations and neighbouring sequence were checked by DNA sequencing. For the yeast two-hybrid assay, fusions to the GAL4 DNA binding and activation domains (BDs and ADs) were constructed by PCR using pET15bFliS, and from derivatives of pET15bFliC (Fig. 2). The PCR products contained 5′ NcoI and 3′ BamHI restriction endonuclease sites, permitting digestion and ligation into the two-hybrid vectors pAS2-1 and pACT2 (Clontech). Recombinant DNAs were confirmed by sequencing.

2.2. Protein analysis and immunodetection

Proteins were resolved by SDS-PAGE using 10%, 12.5% or 15% polyacrylamide gels, stained with Coomassie blue or transferred onto PVDF (Applied Biosystems) or nitrocellulose (Amersham) membranes. For immunoblotting, 3% BSA-PBS-0.5% Tween (PBST) was used for the blocking steps. Rabbit antisera raised against FliC, and goat antisera against GST (Pharmacia) were used at 1:5000 dilutions. Secondary antisera (rabbit (Amersham) or goat (Sigma)) conjugated to horseradish peroxidase were used at 1:10 000 before visualisation with SuperSignal substrate (Pierce). Affinity blotting experiments were performed as described [3].

2.3. Yeast two-hybrid system

The Clontech two-hybrid system was used as described [15] to test pairwise interactions between pAS2-1-FliS and pACT2-FliC derivatives (Fig. 2). Essentially, each of the pACT2FliC plasmids were transformed independently into Saccharomyces cerevisiae Y190 (MATα, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3,112, gal4Δ, gal80Δ, cyh2, LYS2::URA::GAL1UAS-HIS3TATA-HIS3, URA3::GAL1UAS-GAL1TATA-HIS3) and mated with the pAS2-1-FliS-transformed S. cerevisiae Y187 (MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3,112, gal4Δ, met-, gal80Δ, URA3::GAL1UAS-GAL1TATA-HIS3). Diploids containing both plasmids were selected on minimal media lacking leucine and tryptophan, nutritional markers provided by the pACT2-1 and pAS2 vectors respectively. Positive interactions were reported by activation of the GAL4-controlled β-galactosidase gene (colony lift and liquid assays).

2.4. Co-expression and co-puriﬁcation (pull down) assays

Complex formation was analysed by Ni-NTA (Qiagen) affinity chromatography as described [16], using E. coli BL21 (DE3) carrying pET15bFliS and pacT7FliC derivatives (Fig. 3). Pull-down assays using proteins expressed in E. coli BL21 (DE3) carrying pacT7FliS and pGSTFliC derivatives or pGex-4T-3 (Pharmacia), were performed with glutathione-Sepharose resin (Pharmacia).

3. Results

3.1. FliS binds selectively to the disordered C-terminal D0 domain of flagellin

We assayed FliS binding of FliC derivatives by yeast two-hybrid analysis, afﬁnity blotting, and co-puriﬁcation. The results are summarised in Fig. 2, together with representative afﬁnity blots and co-puriﬁcation elutions (Fig. 3A,B).

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Fig. 1. FliC structure [10]. a: Linear map of the morphological domains D0–D3 of the 494-residue FliC, and the 41-kDa protease-protected fragment F41 (residues 56–450, open rectangle) used to determine the FliC crystal structure. The N- and C-terminal disordered regions (residues 56–309 and 310–450) of D0 are depicted by dashed lines. Regular vertical marks indicate 50-amino acid divisions. b: Co backbone of the F41 FliC structure marked with the D1, D2, and D3 domains. The uncrystallised D0 N- and C-termini are represented by dashed lines.
3.1.1. Yeast two-hybrid analysis of interactions between FliS and FliC

Full-length fliS and fliC were fused independently to sequences encoding the GAL4 transcriptional activator BD and AD, in vectors pAS2-1 and pACT2 respectively. The resulting plasmids were introduced into yeast by pairwise mating, and L-galactosidase activity assayed (when AD and BD fusion proteins interact, GAL4-controlled lacZ expression is induced) [17]. No activity was detected when either of the FliS and FliC fusion proteins was co-expressed with the unfused BD or AD domains (not shown), but L-galactosidase activity increased when the fusions were co-expressed, regardless of the combination, i.e. FliS-AD with FliC-BD, or FliS-BD with FliC-AD. Moreover, FliS bound the C-terminal sequence of FliCΔ1–309, but not the C-terminally deleted CΔ410-494 (Fig. 2), confirming the initial indication from affinity blotting [3]. Extensive FliC N-terminal deletion, up to residue 401 (i.e. FliCΔ1–400), did not reduce FliC-AD interaction with FliS-BD, localising the interaction to the last 94 residues. In contrast, removal of as little as 10 residues from the FliC C-terminus (i.e. in the fusion CΔ485-494) abolished interaction (Fig. 2). In further FliC-AD fusion proteins (Fig. 2), deletions N-terminal of residue 454 did not reduce interaction, but activity was reduced four-fold by deletion of residues 454–464 and was abolished in CΔ464-474 and CΔ474-484.


The same truncated and internally deleted FliC sequences were expressed under control of the pET15b T7 promoter in E. coli BL21. FliC proteins in cell extracts were separated by SDS-PAGE and either stained with Coomassie Blue or affinity blotted with 35S-labelled FliS. FliS bound comparably to full-length FliC and N-terminally truncated derivatives up to and including FliCΔ1–400, but did not bind to any of the C-terminally truncated derivatives (Fig. 2). Of the internally deleted variants only CA408–425, CA426–444 and less so CA444.454 were bound by FliS (Figs. 2 and 3A).

3.1.3. Co-purification of FliC by co-expressed histidine-tagged FliS

Histidine-tagged FliS (His-FliS) was co-expressed with untagged wild-type or derivative FliC proteins in E. coli BL21, in every case by low induction of the T7 promoter. In each case, the total amount of expressed protein was comparable (not shown) and soluble His-FliS was readily purified from cell lysates on Nickel-NTA (Ni-NTA) agarose columns with a peak elution fraction between 100 and 300 mM imidazole (Fig. 3B). Untagged full-length FliC had the same elution pattern as His-FliS, and gel filtration and light scattering analysis of the eluted fractions containing His-FliS and FliC confirmed that these proteins co-eluted as a soluble complex (not shown). Comparable

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Fig. 2. Defining the FliS binding region. N- and C-terminal truncations of the 494-residue FliC (upper), and C-terminal truncations and internal deletions in residues 408–494 (lower). Resulting polypeptides were either fused to the GAL4 DNA-AD for two-hybrid assay, or expressed for affinity blotting and co-purification assays. The disordered termini (residues 1–55 and 451–494) are indicated as dashed lines, the F41 central region (residues 56–450) by the open rectangle. GAL4-controlled β-galactosidase activity shows positive interaction (+, 5.5 ± 0.1 Miller units (MU)), weak interaction ([+], 1.2 ± 0.1 MU detected after 1 h by colony lift assays), and no interaction (−, ≤ 0.1 MU).
elution results were obtained with all the derivatives truncated or deleted between residues 1 and 454 (Figs. 2 and 3B). In contrast, FliC derivatives were not retained on the column following deletion between residues 454–494, they were found exclusively in the flow through. The amount of eluted His-FliS was moderately reduced in co-purifications with the non-bound variants (Fig. 3B), this can be attributed to the tendency of His-FliS alone to form insoluble inclusion bodies (not shown), as noted with the wild-type chaperone expressed without FliC [3].

3.2. The disordered C-terminus is sufficient for FliS binding

The three assays show that amino acids 454–494 of FliC are essential for FliS binding, and indicate that no other sequence is involved. To confirm that the C-terminal D0 domain is sufficient for FliS interaction in the absence of the remaining 454 amino acids, GST fusion GST–CA1–454 (Fig. 4) was co-expressed with untagged FliS in E. coli BL21. Cell lysates were subjected to purification on glutathione-Sepharose resin, and after extensive washing, eluted proteins were resolved by SDS-PAGE. The fusion protein captured FliS as detected by Coomassie staining (not shown) and confirmed by immunoblotting with anti-FliS antisera, in contrast to when the unfused GST was co-expressed with FliS (Fig. 4).

![Fig. 3. Defining the FliS binding region. a: Affinity blotting. Cell extracts from E. coli BL21 (DE3) containing vector pET15b alone (–) or expressing wild-type or derivative FliC. Following PAGE, proteins were stained with Coomassie blue (labelled cb), or blotted with 35S-labelled FliS. Markers are in kilodaltons. b: Co-purification of wild-type or derivative FliC (upper) from lysates of E. coli BL21 (DE3) co-expressing His-tagged FliS (lower) by affinity chromatography on Ni-NTA. Flow through (F) and elution (E) from the 300-mM imidazole step-gradient were separated by PAGE and stained with Coomassie blue. Full-length proteins are marked by an asterisk.](https://academic.oup.com/femsle/article-abstract/219/2/219/570560)

![Fig. 4. Minimum region of FliC able to pull down FliS. Soluble extracts of E. coli BL21 co-expressing GST or GST–FliC fusion (upper panel) with FliS (middle panel), separated by SDS–12% PAGE and stained with Coomassie blue. The same soluble extracts were passed over glutathione-Sepharose and affinity-purified proteins were separated by SDS-PAGE and immunoblotted with FliS antisera (lower panel).](https://academic.oup.com/femsle/article-abstract/219/2/219/570560)
3.3. Unchaperoned C-terminal D0 domain is susceptible to proteolysis

We assayed stability of the GST–FliC fusion protein GST–CΔ1–400 (Fig. 2), expressed alone or co-expressed with FliS in E. coli BL21 (DE3). The full-length fusion was detected with both anti-GST (Fig. 5) and anti-FliC antisera (not shown) following SDS-PAGE of whole-cell extracts. In the presence of FliS, there was no FliC degradation, but with no chaperone or with FlgN, a flagellar chaperone specific for the hook associated proteins FlgK and FlgL [18,19], degradation products were evident, about 5kDa smaller than the full-length fusion but larger than the 26-kDa GST moiety (Fig. 5). N-terminal sequencing of the purified degradation products showed that they retained the intact GST N-terminus, so the products arose from cleavage within the FliC sequence, from their size most probably within the protease-sensitive disordered C-terminal region (amino acids 450–494) [8].

4. Discussion

We show that the extreme C-terminal 40 amino acids of flagellin are essential and sufficient for binding by the substrate-specific cytosolic chaperone FliS. The selectivity is significant. Only the N- and C-termini of flagellin are required for polymerisation, and are highly conserved, whereas deletion of the large central region does not prevent assembly [20] and it is not conserved, indeed the flagellin of Caulobacter crescentus lacks it [21,22]. The terminal sequences constitute the disordered D0 domain [8]. FliS prevents polymerisation in vitro [3] and our data further support a view of FliS as a domain-specific ‘bodyguard’, protecting the C-terminal D0 polymerisation domain from inappropriate interaction. As both the N- and C-terminal components of D0 must interact with those of adjacent flagellin monomers during assembly, FliS binding to only the C-terminus is sufficient to control polymerisation. It would also leave free the N-terminus, which contributes to targeting of the monomer to the export apparatus [2,23].

Our view seems compatible with structural analysis of the related type III chaperone SicP bound to a 104 amino acid peptide of its 543-residue virulence substrate SpIP [24], and analysis of chaperone-virulence substrate complexes (E. coli CesT with Tir, and S. typhimurium SigE with SigD, YopE with SycE) that indicate only minimal unfolding of substrates [25,26]. SycE is believed to act primarily as a secretion pilot [26], and type III chaperone structure has been suggested to present a common signal for type III export of bound substrates [27]. Our data do not address the possibility that FliS might also aid targeting to the flagellar export apparatus, and interactions between flagellar chaperone-substrate and membrane components of the flagellar machinery have not been demonstrated. Among type III export substrates flagellin presents the clearest example of a high copy self-interactive substrate that might be expected to benefit from protection by a cytosolic bodyguard. Whether FliS interaction also facilitates translocation is not clear, but it is possible that the targeting aspect has become more prominent in FliS-related chaperones in which non-productive polymerisation is less critical.

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References


