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Signal peptide of FadA adhesin from *Fusobacterium nucleatum* plays a novel structural role by regulating the filament's length and width

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Abstract

FadA, a novel adhesin of periodontal pathogen *Fusobacterium nucleatum* is composed of two forms, pre-FadA and mature FadA (mFadA), constituting the functional FadA complex (FadAc). By electron microscopy, we observed that mFadA formed uniformly long and thin filaments, while FadAc formed heterogeneous filaments of varying lengths and widths, as well as “knots”. Mutants in signal peptide or in the non-alpha helical loop retaining heterogeneous structures had binding activity while those forming aggregates or long filaments lost activity. These observations suggest short filaments and knots may be the active forms of FadA. This is the first demonstration that a signal peptide is required for the assembly of a bacterial adhesin.

1-Introduction

Fusobacterium nucleatum is one of the most abundant gram-negative bacilli colonizing the subgingival plaque (11) and one of the most prevalent species detected in intra-amniotic cavities, associated with pregnancy complications including preterm birth and stillbirth (3, 7). *F. nucleatum* can enter the blood circulation as a result of periodontal infection (dental bacteremia) and spread hematogenously to the pregnant uterus (3). Hematogenous infection of pregnant mice led to colonization of the feto-placental unit and eventually fetal demise (6, 10). *F. nucleatum* adheres to and invades host epithelial and endothelial cells (5, 8). It also promotes the invasion of epithelial cells by non-invasive bacteria (1). Host cell attachment

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Structured summary of protein interactions:
mFadA and pre-FadA bind by transmission electron microscopy (View interaction)

and invasion play a critical role in *F. nucleatum* in intrauterine infection (6, 9). A novel adhesin, FadA, was identified to be involved in *F. nucleatum* attachment and invasion to host cells (4, 5). A *fadA*-deletion mutant, *F. nucleatum* 12230-US1, was defective in host-cell attachment and invasion as well as colonization murine placenta (5). Complementation with FadA restores host-cell adhesion and invasion, and colonization of the placenta (9). FadA is highly conserved among oral fusobacteria species (5). It exists in two forms, the non-secreted pre-FadA, consisting of 129 amino acids, and the secreted mature FadA (mFadA), consisting of 111 amino acids (13) (Supplementary Figure). Pre-FadA possess a signal peptide of 18 amino acids (MKKFLLLAVLAVSASAFA) (5). Pre-FadA is insoluble under neutral pH, unless it is mixed with mFadA (13). An inhibitory cell attachment assay shows that the pre-FadA-mFadA complex (FadAc) inhibited the attachment of *F. nucleatum* to host cells, while mFadA alone did not (13). This observation suggests a potentially novel role of the signal peptide. However, it was unclear how the signal peptide was involved in FadA structure and function.

The crystal structure of mFadA reveals an infinite filament with the monomers linked together in a head-to-tail pattern via a novel leucine chain motif (12). The only non-alpha helical loop is exposed at the tip of the filament. The aim of this study was to examine the role of the non-alpha helical loop region and the signal peptide in the structure and function of FadA. Our results demonstrate and that a proper oligomerization of the FadA protein is essential for its function. It also appears that the signal peptide determines the length and width of the filament and that short filaments and knots may be the active form.

2-Material and Methods

2.1-Bacterial strains and cell lines

E. coli BL21(DE3)-pYWH417-6 carrying the entire *fadA* gene in the pET21(b) expression vector (Novagen) with a His-tag fusion at the carboxyl terminal was used to produce FadAc (13). *E. coli* BL21(DE3)-pYH1490, carrying *fadA* without the signal peptide in pET21(b), was used to produce mFadA alone. *E. coli* strains were cultured aerobically in Luria Bertani broth or agar (BD Diagnostics). *F. nucleatum* 12230 was cultured as described previously (2). For attachment assay, *F. nucleatum* was sub-cultured on the day of the test and allowed to grow anaerobically for 3.5 hr at 37°C. Human Umbilical Vein Endothelial cells (HUVEC) and Chinese Hamster Ovarian (CHO) cells were cultured in F12-K supplemented with 10% fetal bovine serum (Invitrogen) and 100μg/ml Streptomycin-100U/ml Penicillin (Invitrogen). In addition, a total of 30μg/ml Endothelial Cell Growth Supplements (BD Biosciences) and 0.1mg/ml heparin (Sigma) were added for HUVEC and 1mM sodium pyruvate (Mediatech Inc.) for CHO cells.

2.2-Construction of FadA variants

The FadA variants were constructed using the QuickChange®II Site-Directed Mutagenesis Kit (Stratagene) using the primers listed in supplementary Table. Plasmids were purified, verified by PCR, and sequenced at the Genomics Core Facility (CWRU, OH). Correct plasmids were transformed into *E. coli* BL21(DE3) for protein expression.

2.3-Purification and western-blot analysis

Wild-type and variant recombinant FadAc were expressed and purified from *E. coli* BL21(DE3)-pYWH417-6 and its derivatives using denaturing conditions as previously described (13). The mFadA alone was expressed and purified from *E. coli* BL21(DE3)-pYH1490 using either denaturing or native conditions as previously described (13). The protein concentration was determined using the BCA kit (Pierce Biotechnology).

Protein samples were loaded onto 12% SDS-polyacrylamide gels and transferred to Immobilon-Psq PVDF membranes (Millipore). FadA-His tag fusion proteins were detected using FadA monoclonal antibody 5G11-3G8 (13) (1:2000 dilution) and horseradish peroxidase-conjugated goat anti-mouse IgG (1:2000 dilution; Pierce Biotechnology), followed by chemiluminescence using SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology).

2.4-Transmission electron microscopy

A total of 7.5 μg of FadA protein was mounted on Formvar carbon support Nickel grid (Electron Microscopy Science), incubated at room temperature for 5 min and stained with 2% uranyl acetate at room temperature for 1 min. Unmounted proteins and excess dye were removed, and the specimen grid was dried at room temperature for 16 hours. Observation was carried out as previously described, using Jeol JEM-1200EX or Zeiss Libra 200FE electron transmission microscope with 80kV electron acceleration voltage (13).

2.5-Inhibitory cell attachment assays

CHO and HUVEC cells were seeded in 24-well plates (BD Falcon) at 4×10^5 and 8×10^4 cells/well, respectively. FadA proteins (50μg/ml) were pre-incubated on the confluent monolayer for 45 min, prior to the addition of *F. nucleatum* at a multiplicity of infection (MOI) of 5. After one-hour incubation, the monolayers were washed 6 times with PBS pH7.4 (Invitrogen) for CHO cells or 3 times with D-PBS pH7.1 supplemented with Ca^{2+} and Mg^{2+} (Invitrogen) for HUVEC and then lysed with water. Attached bacteria were enumerated by plating serial dilutions on blood agar plates. The level of attachment was expressed as the percentage of bacteria recovered following cell lysis relative to the total number of bacteria initially added. Each experiment was performed in duplicates or more and repeated at least three times.

2.6-Statistical analysis

Attachment assays were analyzed with an analysis of variance followed by a Dunnett's multiple comparison test. These statistical tests were performed with InStat software (GraphPad).

3-Results and Discussion

FadA is a unique protein involved in *F. nucleatum* attachment and invasion of host cells. In this study, we have investigated the role of the non-alpha helical region and the signal peptide in its structure and function.

3.1-Construction and purifications of the FadA mutants

The crystal structure of mFadA has revealed a filamentous structure with the monomers linked in a head-to-tail pattern (12). We surmise that *in vivo* the only exposed non-alpha helical region, i.e. the loop, would be at the tip of the filament, which may be involved in the binding to the host-cell receptor. The crystal structure also showed that the leucine residues are involved in inter- and intra-molecular interactions (12). We speculated that intermolecular interactions involving leucine residues in the signal peptide may also be involved in the proper formation of the FadAc filaments. To test these hypotheses, eight amino-acid residues, E63, N65, T66, R67, F68, Y69, K70 and S71, in the loop region and four in the signal peptide, L-9, L-12, L-13 and L-14, were replaced by alanine by site-directed mutagenesis to avoid disruption of the alpha helical structure, if there is any. Wild-type and mutant proteins were purified by cobalt affinity column chromatography without separating pre-FadA and mFadA, thus producing pre-FadA-mFadA complex (FadAc). Wild-type mFadA was purified from *E. coli* BL21(DE3)-pYWH1490 expressing mFadA alone.

The stability of the mutant proteins was examined by Western-blot. Of all 12 mutants constructed, two in the signal peptide (L-9A and L-12A) and four in the loop region (F68A, Y69A, K70A and S71A) produced stable proteins adequate for analysis. Alanine substitution of the other residues, L-13 and L-14 in the signal peptide and E63, N65, T66 and R67 in the loop region, resulted in no protein recovery even under denaturing conditions although they were detectable in total bacterial extract (data not shown). These mutations may affect the stability of FadA. Alternatively, mutations at L-13 and L-14 may affect FadA secretion. These mutants were not analyzed further.

The stably expressed mutant proteins were purified and analyzed by SDS-PAGE followed by Coomassie blue staining and Western-blot. Little or no pre-FadA was detected from L-12A, F68A, or Y69A by Coomassie blue staining (Figure 1A). However, both pre-FadA and mFadA were detected from all mutants by Western-blot (Figure 1B). This was likely due to that the monoclonal anti-FadA antibodies detected pre-FadA with much higher sensitivity than mFadA as previously observed (13). Compared to the wild-type, L-9A expressed more pre-FadA and less mFadA, whereas L-12A, F68A, and Y69A each expressed less pre-FadA and comparable mFadA (Figure 1B). In K70A and S71A, both pre-FadA and mFadA were expressed at similar levels as the wild type.

3.2-Activity and structure of the mutants

The mutant proteins' activity was analyzed in an inhibitory cell attachment assay using CHO (Figure 2A) and HUVEC cells (Figure 2B) and their structure analyzed by transmission electron microscopy (Figure 3). For the first time, we visualized the striking difference of the filaments formed by FadAc and mFadA. The FadAc filaments were highly heterogeneous, with varying lengths and widths as well as filaments-associated "knots", whereas the mFadA filaments were homogenously long and thin, consistent with what was observed in the mFadA crystal structure. The heterogeneous FadAc filaments exhibited binding activities with an inhibition of the attachment of *F. nucleatum* by approximately 90% to CHO cells and by 70% to HUVEC, while pre-incubation with the homogenous mFadA filaments did not. These results indicate that the signal peptide plays a unique role in FadA structure and function by affecting the length and width (bundling) of the filament.

Similar to the wild-type proteins, L-9A and S71A formed heterogeneous filaments and retained binding activities (Figure 2A) with inhibition of attachment of *F. nucleatum* to CHO cells of 92% and 86%, and to HUVEC of 65% and 71%, respectively. Mutants F68A, Y69A and K70A which exhibited protein aggregates and L-12A which formed fewer and homogeneous filaments, with no short filaments or knots (Figure 3) did not inhibit *F. nucleatum* attachment to CHO and HUVEC cells (Figure 2A), indicating that these mutants have lost binding activity. Although mutants F68A, Y69A and K70A were stably expressed, they were defective in oligomerization and attachment, suggesting that the loop region is required for the structure that is necessary for function. Only those forming heterogeneous filaments similar to the wild type had binding activities highlighting that a correct formation of FadAc is correlated with the function of the protein.

3.3-Titration of pre-FadA required for function of FadA

To determine the amount of pre-FadA required for activity, a titration experiment was performed. Since the proportion of preFadA/mFadA present naturally is not known, wild-type FadAc was mixed with mFadA at varying ratios of 1:2, 1:5, 1:10, 1:25, 1:50, and 1:100 to decrease pre-FadA proportions. We chose to titrate pre-FadA by mixing mFadA and FadAc rather the mixing purified mFadA and pre-FadA due to the difficulty to purify pre-FadA and its insolubility under neutral pH. The resulting mFadA- pre-FadA mixtures were examined by western-blot and cell attachment inhibition assays. Mutants L-12A and F68A,

in which less pre-FadA was detected, were included for comparison. As shown in Figure 4A, attachment inhibition activities decreased with decreasing amounts of pre-FadA in the mixture. At mixing ratios of 1:2, 1:5 and 1:10 (FadAc:mFadA), attachment inhibition activity was comparable to that of the wild-type FadAc. *F. nucleatum* attachment was only inhibited by 40% at 1:25 and not affected at 1:50. The levels of pre-FadA in L-12A and F68A as detected by Western-blot were comparable to that in the mixture of 1:10 (Figure 4B). Thus, the lack of binding of L-12A and F68A was not due to insufficient pre-FadA expressed in these mutants.

The filaments formed by FadAc:mFadA mixtures were also examined by transmission electron microscopy (Figure 5). With decreasing amounts of pre-FadA, the filaments tend to become longer and more homogeneous, with fewer short filaments and knots, and eventually becoming similar to mFadA alone. The FadAc:mFadA mixtures with binding activities (e.g. 1:5 and 1:10) formed heterogeneous structures, with both long and short filaments, as well as knots. At 1:25, very few short filaments were observed. The mixtures with no binding activity (e.g. 1:50) exhibited long filaments, similar to mFadA alone. Thus, the presence of short filaments and filaments-associated knots correlated with activity, suggesting that they may be the active forms of FadA. These observations also indicate that these active forms require pre-FadA.

Interestingly, among the different strains of *F. nucleatum* there is some variations of the pre-FadA:mFadA ratio with more pre-FadA detected in strain 12230 than in strains ATCC-25586 or ATCC-10953 although they all exhibit attachment activities (5, 8). So far, no long filamentous appendages have been reported for *F. nucleatum*, which allows us to speculate that the FadA adhesin may not be exposed on the bacterial cell surface as long filaments. As few as 3 FadA monomers (Supplementary figure) are required to penetrate across the outer membrane. This is consistent with our hypothesis that short filaments and knots are the active forms. The advantage of short and thick filaments may be its strength, which ensures that the bacteria can bind to host cells despite the shearing forces from various body fluids they encounter in the host environment.

In summary, our results highlight the importance of the loop region and the signal peptide in the structure and function of FadA but more importantly that the signal peptide determines the size and shape of the filament. To the best of our knowledge, this is the first demonstration of a signal peptide being involved in the assembly of a bacterial adhesin. A correct oligomerization is required for the function of FadA but there is some flexibility in the ratio of pre-FadA:mFadA to retain structure and function. The mode by which the signal peptide modulates the FadA structure is currently under investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

1. -mFadA and FadAc have distinct structures observed under EM.
2. -mFadA forms uniformly long and thin filaments which have no binding activity
3. -FadAc forms heterogeneous structures, due to the presence of pre-FadA
4. -The heterogeneity of the FadA structure correlates with function
5. -The shorter filaments may be the active form of FadA
6. -This is the first demonstration that signal peptide may be involved in adhesin assembly

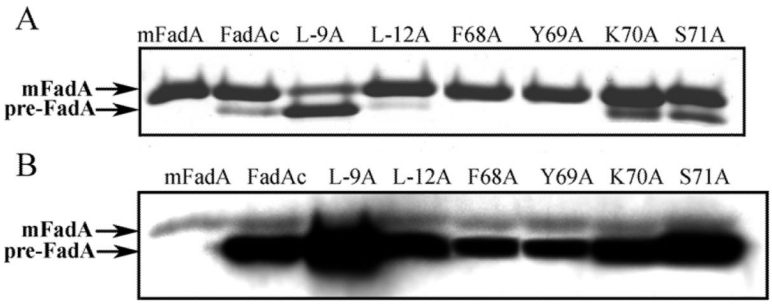

Figure 1.

Analysis of mutated FadA. The pre-FadA and mFadA were co-purified and 3 μg of proteins per lane were analyzed by Coomassie Blue staining (A) or 2 μg by Western-blot with FadA monoclonal antibody Ab5G11-3G8 (B). The migration of FadA on SDS-PAGE is abnormal as the larger pre-FadA migrates faster than the smaller mFadA (13).

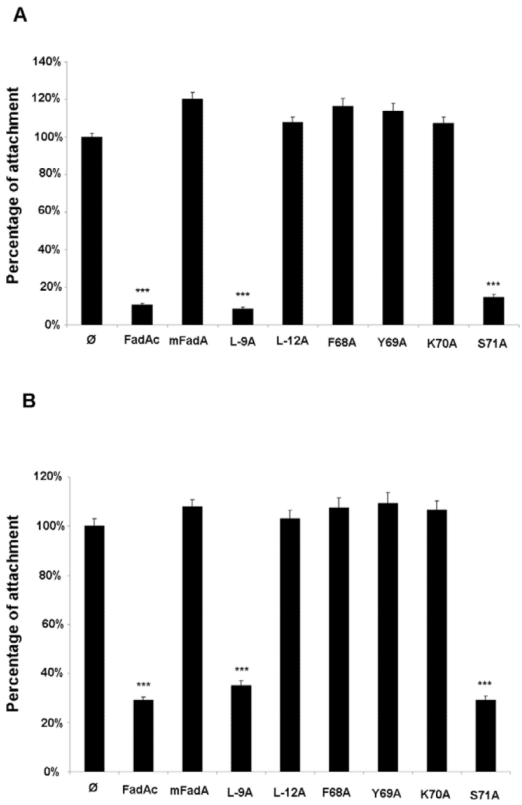

Figure 2.

Cell-attachment inhibition assay using CHO (A) and HUVEC cells (B). A total of 50μg/ml of FadA proteins were incubated for 45 min prior to the addition of *Fusobacterium nucleatum* to the confluent monolayers at a MOI of 5. After 1 hour incubation, cells were washed, lysed and a viable count was performed on attached bacteria. Total cell-associated *F. nucleatum* was arbitrary set to 100% and that in the presence of the proteins was compared to it. Cell attachment inhibition assays results represent an average of three experiments performed at least in duplicate.

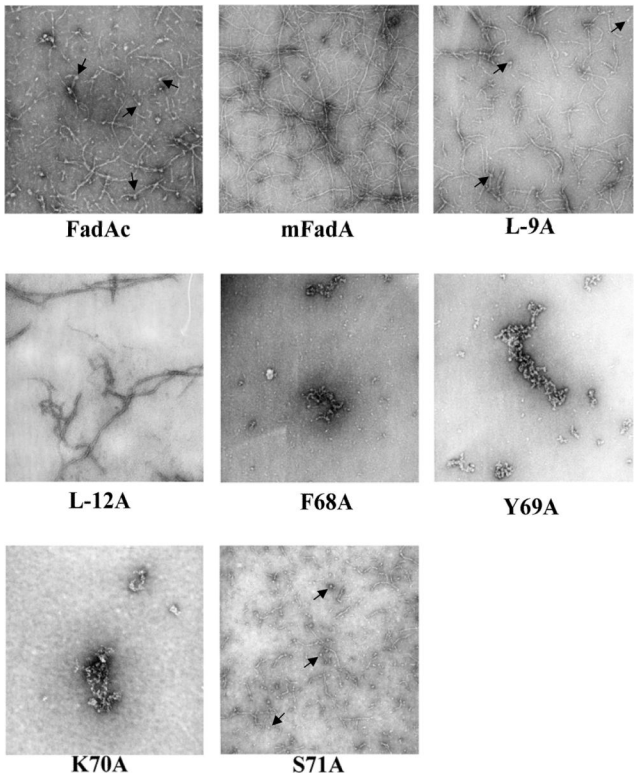

Figure 3.

Transmission electron microscopy pictures of FadA mutants proteins (7.5μg) mounted on grid and stained with 2% uranyl acetate using Jeol JEM-1200EX microscope. Magnification × 50000. Short filaments and knots (arrows) present in FadAc, S71A and L-9A were absent in other samples.

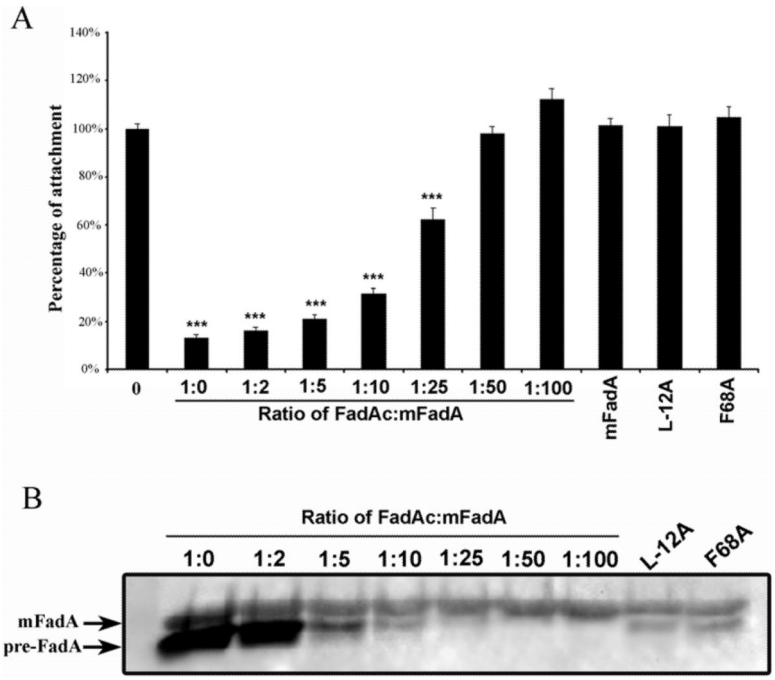

Figure 4.

Analysis of pre-FadA involvement in cell-attachment inhibition assay on CHO cells (A) and by Western-blot (B). A total of 50 μg/ml of FadA proteins were pre-incubated on cells for 45 min, before *F. nucleatum* was added at a MOI of 5. Cell attachment inhibition assays results represent an average of three experiments performed in duplicate (A). A total of 5 μg of FadAc, FadAc mutants, or mixtures of FadAc:mFadA at different ratios were loaded per lane. Proteins were analyzed by Western-blot using FadA monoclonal antibody Ab5G11-3G8 (B).

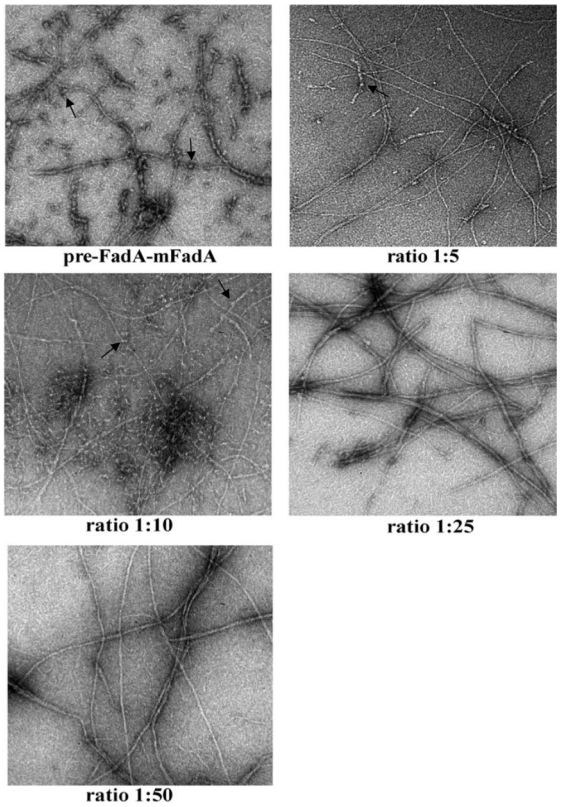

Figure 5.
Transmission electron microscopy pictures of different mixing ratio of FadAc:mFadA (7.5μg) using Zeiss Libra 200FE microscope. Magnification × 25000.Short filaments and knots (arrows) present in FadAc and 1:5 and 1:10 mixtures, but not in other samples.