Molecular Basis of the Interaction between the Flagellar Export Proteins Flil and FlIH from *Helicobacter pylori*

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Bacterial flagellar protein export requires an ATPase, Flil, and presumptive inhibitor, FlIH. We have explored the molecular basis for Flil/FlIH interaction in the human gastric pathogen *Helicobacter pylori*. By using bioinformatic and biochemical analyses, we showed that residues 1–18 of Flil very likely form an amphipathic α-helix upon interaction with FlIH, and that residues 21–91 of Flil resemble the N-terminal oligomerization domain of the F1-ATPase catalytic subunits. A truncated Flil (2–91) protein was shown to be folded, although the N-terminal 18 residues were likely unstructured. Deletion and scanning mutagenesis showed that residues 1–18 of Flil were essential for the Flil/FlIH interaction. Scanning mutation of amino acids in the N-terminal 10 residues of Flil indicated that a cluster of hydrophobic residues in this segment was critical for the interaction with FlIH. The interaction between Flil and FlIH has similarities to the interaction between the N-terminal α-helix of the F1-ATPase α-subunit and the globular domain of the F1-ATPase δ-subunit, respectively. This similarity suggests that FlIH may function as a molecular stator.

*Helicobacter pylori* is a Gram-negative microaerophilic bacterium that colonizes the human gastric mucosa and is associated with a number of gastric diseases (1–4). *H. pylori* motility is conferred by the production of multiple polar flagella and is essential for colonization and persistence by the pathogen (5, 6). The study of *H. pylori* flagellar protein export and flagellum assembly is relatively new; most of our knowledge of the bacterial flagellum comes from studies in *Salmonella enterica* serovar Typhimurium (7, 8). Comparison of known *Salmonella* flagellar genes with the *H. pylori* genome confirms the existence of *H. pylori* homologs for most flagellar proteins in *Salmonella* and *Escherichia coli* (9, 10). However, the regulation of several of these genes appears to be distinct from the *Salmonella* paradigm (10, 11). A two-hybrid study of *H. pylori* protein/protein interactions revealed a small number of expected flagellar protein interactions (12). Flagellar proteins not annotated in the *H. pylori* genome include regulators such as FlhC, FlhD, and FlIK and the chaperones FljL, FlgN, and FlgT. The distinctive regulation of *H. pylori* flagellar gene expression (9–11), the flagellar configuration (polar, sheathed), and the absolute requirement of motility for *H. pylori* pathogenesis make flagellar biogenesis an attractive candidate for molecular studies in this organism.

Flagellum secretion machines (7, 13–15) contain a highly conserved flagellum-specific ATPase, Flil, essential for the secretion of flagellar hook and filament proteins. Flil and other export apparatus proteins are thought to associate with the flagellar basal body at the cell membrane (7, 16–23). Flil from *Salmonella* is a 456-amino acid polypeptide that peripherally associates with the bacterial cytoplasmic membrane (22, 23). In the presence of ATP, *Salmonella* Flil forms hexamers and demonstrates positive cooperativity of ATP hydrolysis *in vitro*, and both activities are stimulated in the presence of anionic phospholipids (23). *Salmonella* Flil has also been shown to interact with FlIH, a conserved flagellar export component that inhibits Flil enzymatic activity *in vitro* (24–28).

Several studies (16–18, 26) suggest that *Salmonella* Flil contains two domains, a poorly characterized N-terminal segment (amino acids 1–97) with secretion-specific functions and a C-terminal catalytic domain (amino acids 100–456) homologous to the catalytic domains of the α- and β-subunits of F1-ATPase. Mutation of active site residues in the C-terminal domain of Flil are dominant negative for swarming motility (16–18). Similar studies on the homologous InvC type III ATPase also demonstrated negative dominance of catalytic site mutations that were relieved on disruption of membrane localization of the ATPase (20, 21).

The interaction of Flil with FlIH, a conserved component of the flagellar and type III secretion systems that inhibits Flil ATPase activity *in vitro*, has been studied in *Salmonella* (24–27). An N-terminal Flil double mutant (R7C/L12P) was isolated from genetic screens that detected loss of swarming motility *in vivo* and acted in a recessive manner (26). This double mutant also failed to interact with FlIH *in vitro* (26), suggesting individual residues in this segment of Flil were required for interaction with one or more components of the export apparatus, including FlIH. However, further experiments were not carried out to verify and substantiate this preliminary observation. Furthermore, the extreme nature of the R7C/L12P double mutant suggested this mutation very likely prevented the Flil N terminus from folding into a structure capable of interacting with FlIH. Nevertheless, truncated versions of *Salmonella* Flil purified from limited proteolysis experiments and containing residues 7–456 or 26–456 did not interact with FlIH as judged by gel filtration chromatography (24), indicating the N terminus of Flil is largely responsible for interactions with FlIH. These studies also indicated that *Salmonella* Flil was sensitive to clostripain proteolysis at amino acids 7, 26, 93, and 97, but the extent of Flil proteolysis was reduced in the presence of *Salmonella* FlIH (24). FlIH itself was not sensitive to clostripain proteolysis.

The domain structure of *Salmonella* FlIH has been studied in some detail. *Salmonella* FlIH forms an elongated dimeric structure in solution...
hand a (FliH), Flii complex forms with intact full-length Flii (27). A scanning deletion analysis of Salmonella Flii showed that residues 100–235 were required for interactions with Flii; residues 101–141 were required for Flii dimerization (likely via a coiled-coil segment); residues 70–100 were important for inhibition of Flii ATPase activity, and N-terminal residues contributed to binding to the flagellar chaperone Flij (28). However, these studies do not suggest how Flii inhibits Flii ATPase activity, or what is the general role of Flii in flagellar protein secretion.

Our laboratories are studying flagellar biogenesis in the human gastric pathogen H. pylori as a model for the assembly of a polar sheathed flagellum (10, 19, 29, 30). Thus, we have recently identified the hook length control protein Flik (29) and a novel component that interacts with RpoN and Flii that is essential for flagellum assembly (30). We are also investigating individual flagellar components. The principal objective of this study was to extend our understanding of Fli structure and function based on analysis of the poorly characterized yet important N-terminal region of the molecule (residues 1–91 of H. pylori Flii). Furthermore, we hoped to gain insight into Fli function from analyzing the molecular details of the Fli/Flii interaction. A previously published Y2H partial interaction map of the H. pylori proteome demonstrated that a bait domain containing residues 1–258 of H. pylori Flii interacted with a prey domain containing residues 3–134 of H. pylori Flii (see Ref. 12 and pimbyribigenics.com). However, these studies did not indicate what residues in this fragment of H. pylori Flii were responsible for the interaction with Flii and, as in the Salmonella studies, did not indicate the molecular nature or absolute residue requirements of the interaction. Hence, biochemical confirmation and elaboration of the H. pylori Flii/Flii Y2H interaction (12) were necessary. In addition, no obvious sequence similarity exists between amino acids 1–25 of Salmonella Flii (reported to be required for Flii interaction) and the same region of H. pylori Flii or other Flii sequences.

MATERIALS AND METHODS

Protein Sequence and Structure Analysis—PSI-BLAST searches (31, 32) for Flii homologs were conducted with standard parameters (www.ncbi.nlm.nih.gov/BLAST) using the H. pylori 26695 Flii protein sequence (HP1420; GenBank™ accession number 15646029). A selected group of Flii, type III ATPase, and F1-ATPase α- and β-subunit sequences uncovered with the PSI-BLAST search were then prepared as a multiple sequence alignments with T-Coffee (supplemental Fig. S1) (33). In addition, three-dimensional structures are available for the bovine mitochondrial and PS3 F1-ATPase (25.7% identity with respect to the respective oligomerization states of purified truncated Flii and FliH proteins, they were further purified by anion exchange chromatography and a novel component that interacts with RpoN and Flii that is essential for flagellum assembly (30). We are also investigating individual flagellar components. The principal objective of this study was to extend our understanding of Fli structure and function based on analysis of the poorly characterized yet important N-terminal region of the molecule (residues 1–91 of H. pylori Flii). Furthermore, we hoped to gain insight into Fli function from analyzing the molecular details of the Fli/Flii interaction. A previously published Y2H partial interaction map of the H. pylori proteome demonstrated that a bait domain containing residues 1–258 of H. pylori Flii interacted with a prey domain containing residues 3–134 of H. pylori Flii (see Ref. 12 and pimbyribigenics.com). However, these studies did not indicate what residues in this fragment of H. pylori Flii were responsible for the interaction with Flii and, as in the Salmonella studies, did not indicate the molecular nature or absolute residue requirements of the interaction. Hence, biochemical confirmation and elaboration of the H. pylori Flii/Flii Y2H interaction (12) were necessary. In addition, no obvious sequence similarity exists between amino acids 1–25 of Salmonella Flii (reported to be required for Flii interaction) and the same region of H. pylori Flii or other Flii sequences.

Overproduction and Purification of N-terminally GST-tagged Proteins—GST fusion proteins containing the truncated proteins Flii-H(2–258), Flii-H(55–258), Flii-H(94–258), Flii-H(117–258), Flii-(19–91) Flii-(2–91), and the eight Flii-(2–91) point mutants were purified from the soluble fractions of E. coli Rosetta cells according to the guidelines from Amersham Biosciences for the purification of GST fusion proteins. In brief, the cells were grown at 37 °C to an A600 between 0.4 and 0.6 and induced with 0.1 mM isopropyl β-D-thiogalactoside. Following induction, the cells were grown at 25 °C overnight. Cells were harvested, frozen overnight, and lysed by a French press. The supernatant was clarified by centrifuging at 13,000 × g twice for 30 min and then incubated with glutathione-Sepharose for 3 h at 25 °C. The GST affinity tag was removed by adding PreScission protease to the glutathione-Sepharose-bound fusion protein or by adding protease once reduced glutathione was used to release the fusion protein from the resin. For the Flii proteins, they were further purified by anion exchange chromatography on Source Q resin at pH 8.5 followed by gel filtration chromatography in phosphate-buffered saline (PBS) buffer using a Superdex 200 analytical gel filtration column. Flii-(19–91) was purified on Source S followed by gel filtration on Superdex 75. Flii-(2–91) and mutants thereof were purified by glutathione-Sepharose affinity chromatography followed by hydrophobic interaction chromatography (HiTrap phenyl-HP) and finally gel filtration chromatography on Superdex 75.

Molecular Mass Estimation of Purified Proteins—Analytical gel filtration chromatography was used to estimate the molecular masses and respective oligomerization states of purified truncated Flii and FliH proteins. Analytical grade Superdex 75 and Superdex 200 columns from Amersham Biosciences were calibrated with a set of molecular mass standards. The calibration curve plotted is as follows: Kav = (Ve – V0) / (Vt – V0) versus the log10(molecular mass), where V0 is the sample elution volume; Vt is the total bed volume; and V0 is the column void volume. A line of best fit was fitted against the data points. The Superdex 75 column had a void volume of 7.84 ml determined from the elution of blue dextran, and the total bed volume was 24 ml. The Superdex 75 column was calibrated with the following standards: albumin (Mw = 67,000, Ve = 9.84 ml), ovalbumin (Mw = 43,000, Ve = 10.88 ml), chymotrypsinogen A (Mw = 25,000, Ve = 12.80 ml), ribonu-

3 The abbreviations used are: GST, glutathione S-transferase; PBS, phosphate-buffered saline; HSQC, heteronuclear single quantum coherence.
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clease A ($M_c = 13,700$, $V_c = 13.80$ ml), aprotinin ($M_c = 6,500$, $V_c = 15.73$ ml), and vitamin B$_{12}$ ($M_c = 1355$, $V_c = 19.15$ ml).

The Superdex 200 column had a void volume of 8.04 ml determined from the elution of blue dextran, and the total bed volume was 24 ml. The Superdex 200 column was calibrated with the following standards: ferritin ($M_r = 440,000$, $V_c = 9.12$ ml), catalase ($M_r = 232,000$, $V_c = 10.64$ ml), aldolase ($M_r = 158,000$, $V_c = 12.40$ ml), albumin ($M_r = 67,000$, $V_c = 14.00$ ml), ovalbumin ($M_r = 43,000$, $V_c = 15.12$ ml), chymotrypsinogen A ($M_r = 25,000$, $V_c = 16.80$ ml), and aprotinin ($M_r = 6,500$, $V_c = 19.01$ ml).

GST Pulldown Assays—30 g of glutathione-Sepharose was prepared by applying four washes of 3 volumes of PBS buffer and then mixing with 20 g of GST–Filii (2–258), GST–Filii (55–258), GST–Filii (94–258), GST–Filii (117–258), or GST (all in PBS) for 30 min at room temperature. The protein-bound glutathione-Sepharose was then washed three times with 3 volumes of PBS. Then 4.8 g of the purified Filii (19–91), Filii (2–91), or mutant Filii (2–91) proteins was added to yield a 2:1 molar ratio of GST–Filii to Filii. The total volume was made to 200 ml with PBS. This mixture was allowed to incubate with gentle agitation for 30 min. The protein-bound glutathione-Sepharose was then washed twice with 200 ml of PBS, separated by centrifugation, and boiled with SDS-PAGE loading buffer. The proteins were visualized with Coomassie Brilliant Blue on 15% SDS-polyacrylamide gels. Each well of the gel series was repeated in triplicate and shown to be reproducible. Control pulldowns for all of the purified prey proteins bound significantly to glutathione-Sepharose.

Limited Proteolysis of Proteins—Trypsin or chymotrypsin was dissolved to a concentration of 0.1 mg/ml in 10% glycerol and PBS. Protease was added to 50 g of the protein of interest (Filii (2–91) or Filii (2–258)) at a protease to target molar ratio of 1:1000, and the reaction was allowed to proceed for 60 min at room temperature. 10 g of protein were removed after 5, 10, 20, or 40 min and quenched with phenylmethylsulfonyl fluoride. The protein was visualized with Coomassie Brilliant Blue on SDS-polyacrylamide gels. Bands corresponding to protease digestion products were electroblotted onto polyvinylidene fluoride membranes and then subjected to micro-sequencing. We typically sequenced at least five residues to determine the position of protease cleavage. We also micro-sequenced several of the purified proteins used in this study to verify that their N termini were intact.

RESULTS

The N Terminus of H. pylori Filii Is Homologous to the F$_1$-ATPase Oligomerization Domain—Previous studies have established significant amino acid sequence similarity between residues 100–430 of Filii and the catalytic domain of the F$_1$-ATPase $\beta$-subunit, indicating that the catalytic segments of these proteins descended from a common ancestor, and have a similar three-dimensional structure (16–18, 21). However, the N-terminal domains of both Filii and the F$_1$-ATPase catalytic subunits are also similar in size (~100 amino acids), and this prompted us to ascertain if the evolutionary relationship between these proteins extends to their respective N-terminal domains. PSI-BLAST searches (31, 32) of the nonredundant protein sequence data base demonstrated that residues 20–90 of H. pylori Filii are homologous to the oligomerization domain found in both the $\alpha$- and $\beta$-subunits of F$_1$-ATPase (E-value scores ranging from $10^{-165}$ to $10^{-158}$). The alignments span residues 20–430 of Filii and residues ~20–430 of either the $\alpha$- or $\beta$-subunits of F$_1$-ATPase. These results are summarized in a multiple sequence alignment containing Filii and type III ATPase sequences and F$_1$-ATPase $\alpha$- and $\beta$-subunit sequences whose three-dimensional structures are known (34, 36) (supplemental Fig. S1). There are several invariant glycine residues in the alignment and a number of well conserved hydrophobic residues. The alignment was checked by building a model of residues 20–91 of H. pylori Filii using the structure of Bacillus PS3 F$_1$-ATPase as the template (Fig. 1). The modeled structure indicated the presence of a conserved hydrophobic core in the predicted $\beta$-barrel domain and that this domain is easily assembled into a hexameric ring structure based on the hexameric arrangement of the F$_1$-ATPase subunit oligomerization domains in the F$_1$-ATPase structure (34, 36). Independent secondary structure predictions of residues 20–90 of Filii (results not shown) also substantiate a $\beta$-sheet structure for this segment. We also calculated the electrostatic surface potential of the model coordinates for residues 19–91 of hexameric H. pylori Filii (Fig. 1) because the Filii N-domain should be capable of interacting with anionic membrane phospholipids (21–23). The modeled structure does indeed exhibit an electropositive surface potential on the surface most likely to contact the cell membrane (Fig. 1). Hence, we concluded that our alignment and structural modeling for residues 20–90 of Filii reliably predicts a $\beta$-barrel structure for the N-domain of Filii.

Residues 1–18 of H. pylori Filii Likely Form an Amphipathic $\alpha$-Helix—Residues 1–18 of H. pylori Filii are poorly conserved at the amino acid sequence level when compared with other Filii N-terminal sequences (supplemental Fig. S1) but do have a similar composition enriched in nonpolar and positively charged amino acids. Significantly, hydrophobic amino acids in the N-terminal segment of Filii have a similar periodicity to hydrophobic residues in the N-terminal segment of the F$_1$-ATPase $\alpha$-subunit that is known to form an amphipathic $\alpha$-helix (supplemental Fig. S1) (42). The amphipathic nature of the Filii N-terminal segment suggests this segment is likely to form a short amphipathic $\alpha$-helix (Fig. 1D). Secondary structure prediction indicated that residues 3–12 of H. pylori Filii have a high likelihood of forming an amphipathic $\alpha$-helix. Other Filii N-terminal sequences also exhibit a high probability of helix formation (70–80%). Helical wheel plots of Filii N-terminal sequences, including those of H. pylori and Salmonella (shown in Fig. 1D), are highly suggestive of an amphipathic $\alpha$-helix. These data demonstrate that the Filii N-terminal sequences are consistent with an amphipathic $\alpha$-helical structure. The hydrophilic face of the predicted Filii N-terminal helix would exhibit a positive electrostatic surface, enabling it to potentially interact with anionic membrane lipids. Residues 13–18 of H. pylori Filii likely form a flexible loop, connecting the N-terminal helix to the predicted $\beta$-barrel domain.

Expression and Characterization of Filii (2–91) and Filii (19–91)—To study the N-terminal domain structure of H. pylori Filii and to verify yeast two-hybrid interactions with Filih (12), we cloned a gene fragment corresponding to residues 2–91 of Filii (HP1420) from H. pylori 26695.
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genomic DNA as a GST fusion construct. The cloning and expression of truncated FliI N-terminal domains has not been attempted previously. GST fusion constructs of FliI-(2–91) purified from E. coli lysates were soluble and were purified to homogeneity (Fig. 2). The purified N-domain of FliI was deduced to be mostly folded from limited proteolysis experiments (Fig. 2) conducted in the presence of trypsin, although residues 2–18 were easily removed by trypsin treatment, as subsequent protein microsequencing of the major proteolysis product revealed a single cleavage site before Arg-19. In addition, the $^{1}H-^{15}N$ heteronuclear single quantum coherence (HSQC) NMR spectra of a $^{15}N$ isotopically labeled FliI-(2–91) fragment demonstrated good chemical shift dispersion for the majority of the backbone amide N-H peaks. However, ~20 of the amide N-H peaks exhibited a narrow chemical shift distribution characteristic of a random coil structure. Far-UV circular dichroism analysis of FliI-(2–91) also indicated a considerable degree of folded structure (Fig. 2). But far-UV CD spectra of a synthetic peptide containing residues 2–14 of H. pylori FliI indicated a random coil structure.

Therefore, we proposed that residues 1–18 of H. pylori FliI likely correspond to the ~20 random coil peaks in the $^{1}H-^{15}N$ HSQC spectrum and hence are unstructured in solution.

Analytical gel filtration profiles indicated that FliI-(2–91) eluted in two distinct peaks, indicating a mixture of FliI oligomers in solution. SDS-PAGE analysis of the gel filtration peak fractions verified that the same protein comprised each peak from the gel filtration experiments (results not shown). The elution volumes of the two FliI-(2–91) species on Superdex 75 corresponded to the molecular masses of 58.9 and 14.9 kDa, respectively, when compared against the elution volumes of several protein standards of known molecular mass (Fig. 2; see "Materials and Methods"). Repeating the same experiments on Superdex 200 (Fig. 6B) yielded two peaks with elution volumes consistent with molecular masses of 43.6 and 15.5 kDa. If we average the FliI-(2–91) molecular mass values determined from the two gel filtration experiments, then the predicted masses of the FliI-(2–91) oligomers observed in these two experiments are 51 and 15 kDa, respectively. As there is little doubt that the 15-kDa peak represents an FliI-(2–91) monomer (the amino acid sequence predicts a molecular mass of 10.3 kDa), the 51-kDa peak very likely corresponds to a trimer of FliI-(2–91) molecules. It is also not surprising that the apparent molecular mass of FliI-(2–91) predicted from gel filtration (15 kDa) is larger than the molecular mass predicted from the amino acid sequence (10.3 kDa) as residues 2–18 of FliI appear to be largely unstructured in solution and likely exist in an extended conformation. The fact that FliI-(2–91) appears capable of forming a trimcric structure in vitro may have implications for in vivo FliI assembly. Native full-length Salmonella FliI is known to form hexamers (23). We propose that the FliI-(2–91) and FliI-(19–91) trimers (see below) observed here likely correspond to one-half of a completely assembled FliI hexamer and hence represent an initial step in FliI hexamer assembly.

Because FliI-(2–91) was sensitive to limited proteolysis, we concluded that we could express a discrete domain of FliI containing residues 19–91, and this was verified by cloning, protein expression, and purification (Fig. 2). Again, we estimated the molecular mass of the FliI-(19–91) species by using both Superdex 200 and Superdex 75 analytical gel filtration media (Figs. 2C and 7B). In each case, FliI-(19–91) eluted as a single peak with a predicted molecular mass of 30 kDa. The calculated molecular mass of FliI-(19–91) is 8.3 kDa, and hence we concluded that FliI-(19–91) is also most likely a trimer in solution. The FliI-(19–91) domain was later used as a control in verifying interactions between FliI and FliH.

4 H. Iwai, M. Lane, and S. Moore, unpublished data.
5 M. Lane and S. Moore, unpublished results.

**FIGURE 1**. Structural model of the H. pylori FliI N-domain residues 21–91 based on the alignment in Fig. S1. A, C-a coil trace drawn with Molscript-Raster3D (45, 46) is shown depicting the amino acid side chains of the hydrophobic core. Side chains are colored green for aliphatic amino acids, purple for proline, and gold for aromatic amino acids. B, ribbon diagram of the proposed H. pylori FliI N-domain hexamer, based on the arrangement of subunits in F$_{1}$-ATPase. Drawn with Molscript-Raster3D (45, 46). C, electrostatic surface of the modeled FliI N-domain hexamer. Negative potential is red and positive potential is blue. Drawn with GRASP (47). D, helical wheel representation of the N-terminal sequences for H. pylori (starting at residue 2) and S. enterica FliI (starting at residue 5).
Characterization of Truncated FliH-(94–258) and FliH-(117–258) Proteins—Full-length *H. pylori* FliH (HP0353) was cloned by PCR amplification from *H. pylori* 26695 genomic DNA into a GST fusion protein expression vector. The full-length protein aggregated during subsequent attempts at purification. Hence, we pursued the identification of nonaggregative truncated FliH proteins to facilitate the study of interactions with FliI-(2–91). FliH-(55–258) was generated by limited trypsin digestion of full-length *H. pylori* FliH, and subsequent electroblotting and microsequencing of the major digestion products identified the N-terminal sequence of the major cleavage fragment. Upon further characterization and purification, it was evident that FliH-(55–258), although soluble and capable of interacting with FliI-(2–91) reproducibly, did not elute quantitatively from the gel filtration media. We therefore recloned N-terminally truncated versions of *H. pylori* FliH based on sequence conservation with other members of the FliH family. Two fragments were produced that should be capable of binding FliI, based on studies in *Salmonella* (28). These truncations are FliH-(94–258) and FliH-(117–258). Both truncated FliH proteins were purified to homogeneity (Fig. 2) and shown to be folded and monodisperse by analytical gel filtration and CD spectroscopy, respectively (Fig. 3). The far-UV CD spectra of FliH-(94–258) and FliH-(117–258) indicated

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S. Moore, unpublished results.
that both proteins exhibited a high proportion of secondary structure, especially $\alpha$-helix (Fig. 3B). However, \textit{H. pylori} FliH-(117–258) had less helical content than FliH-(94–258) according to the magnitude of the trough at 215 nm in the CD spectra (Fig. 3B). In addition, the gel filtration elution volumes of the truncated FliH proteins were consistent with FliH molecular masses of $\sim$58 or 45 kDa, respectively, based on calibration of the column with molecular standards of known mass (Fig. 3A; see "Materials and Methods"). The molecular masses of the FliH-(94–258) and FliH-(117–258) molecules derived from the amino acid sequences are 18.9 and 16.2 kDa, respectively. Hence, each of the truncated FliH molecules could exist either as trimers (56.7 and 48.6 kDa, respectively) or dimers (37.8 and 32.4 kDa, respectively). Although our gel filtration profile data taken alone are most consistent with a trimer of FliH molecules existing in solution, previous studies have shown that \textit{Salmonella} FliH has a highly asymmetric dimeric structure and hence elutes on gel filtration media at volumes much smaller than those predicted by its apparent molecular mass and known dimeric structure (24, 26, 27). However, the truncated \textit{H. pylori} FliH molecules used in this study are significantly smaller than \textit{Salmonella} FliH (141 and 164 amino acids versus 235 amino acids) and may be considerably less aspherical in solution. We tentatively concluded that the solution structure of truncated H. pylori FliH is most likely an elongated dimer that migrates as an anomalously large species on gel filtration media (see "Discussion").

\textit{FliI-(2–91), but Not FliI-(19–91), Interacts with FliH}—When tested for interactions with the four truncated FliH proteins (full-length FliH and FliH fragments 55–258, 94–258, and 117–258), FliI-(2–91) interacted with all of them by GST pulldown assay, and FliI-(19–91) interacted with none of them (Fig. 4, and data not shown). This indicated that the major if not sole determinant for interactions with FliH resided within the first 18 residues of \textit{H. pylori} FliI. This provided biochemical confirmation of the FliI/FliH interaction in \textit{H. pylori} and validated the published \textit{H. pylori} Y2H data (12).

We then investigated the FliI N-terminal sequence of 18 residues that allowed FliI-(2–91) to interact specifically with FliH. Because we have predicted that this segment of FliI is capable of forming an amphipathic $\alpha$-helix, we hypothesized that a hydrophobic surface from such an FliI N-terminal helix could be the basis for interactions with FliH. We tested this by mutating most of the hydrophobic and positively charged amino acids to alanine or glutamate in residues 2–10 of \textit{H. pylori} FliI. The proteins corresponding to these eight mutations were then expressed, purified, characterized, and tested for interactions with recombinant FliH (Fig. 5). GST pulldowns were conducted by using equimolar ratios of GST-FliH and each of the respective purified FliI-(2–91) mutant proteins. The GST pulldowns were highly reproducible, and the FliI-(2–91) mutants were then tested with each of the truncated FliH-(55–258), FliH-(94–258), and FliH-(117–258) proteins. All three truncated FliH proteins yielded essentially identical results with the purified FliI-(2–91) mutant proteins (FliH-(55–258) and FliH-(94–258) are shown in Fig. 5). Mutation of Leu-3 to Ala (L3A), Leu-6 to Ala (L6A), or Leu-10 to Ala (L10A) in FliI-(2–91) resulted in a dramatically weakened FliI-(2–91)/FliH interaction. A more drastic change of Leu-6 to Glu (L6E) also disrupted the interaction with FliH and was reproducibly weaker than the Leu to Ala mutations (Fig. 5). Additionally, we made Arg-9 to Ala (R9A), Arg-9 to Glu (R9E), Lys-7 to Ala (K7A), and Lys-4 to Ala (K4A) mutations and tested these for interaction with FliH. R9A, K7A, and K4A mutations interacted as well as wild type FliI-(2–91) with the recombinant FliH proteins (Fig. 5). However, a more drastic Arg-9 to Glu (R9E) mutation significantly weakened the FliI/FliH interaction. Hence, FliI principally interacts with FliH via three leucine residues at positions 3, 6, and 10 in the FliI sequence.

To ensure that the mutant Fli proteins had similar physical and chemical properties to the wild type FliI-(2–91), we examined each of the purified FliI mutants for their oligomerization properties as measured by elution profiles on a Superdex 75 analytical gel filtration column (Fig. 2B). The elution profiles of wild type FliI-(2–91) and the eight tested FliI N-terminal point mutants indicated that the mutant proteins behaved essentially the same as wild type FliI-(2–91) and eluted as a mixture of two peaks (assumed to be trimer and some monomer), although the relative ratio of the two peaks depended on the concentration of loaded protein. Far-UV CD spectra of the FliI-(2–91) point mutants were also indistinguishable from wild type FliI-(2–91).\textsuperscript{5} Hence, we concluded that the FliI N-terminal point mutations did not appreciably alter the structure of the FliI-(2–91) protein.

We then demonstrated that the FliI-(2–91):FliH-(117–258) complex is very stable in solution and can be isolated and purified by gel filtration chromatography (Fig. 6). The complex appears to have a 1:1 ratio of FliI-(2–91) and FliH-(117–258) by SDS-PAGE analysis of the peak associated with the eluted complex (Fig. 6). The ratio of proteins visualized on the gel was verified by Coomassie staining of SDS gels of mixtures of known quantities of FliH and FliI (not shown). However, the elution volume of the FliH-FliI complex on Superdex 200 suggests a molecular
mass of $1.3 \times 10^5$ Da, indicating that more than one copy of FliI-(2–91) and FliH-(117–258) is in the complex. Hence, the stoichiometry of the FliI-FliH complex appears to contain two FliH dimers along with three copies of FliI-(2–91), but this will have to be verified with other experimental approaches. The gel filtration peak associated with the FliI-FliH complex was collected, concentrated, and re-injected onto the Superdex 200 column and showed negligible dissociation into FliI-(2–91) and FliH-(117–258) during elution, indicating that this molecular association is very stable (Fig. 6). This complex does not form when the FliI-(2–91) L3A mutant is incubated with FliH-(117–258) or when FliI-(19–91) is incubated with FliH-(117–258) (Fig. 7).

**DISCUSSION**

The purpose of this study was to improve our understanding of FliI structure and function by biochemically characterizing its N-terminal domain and to elucidate the detailed nature of the molecular basis of the interaction FliI with FliH. Our results support the view that the N terminus of FliI is organized into two functional regions as follows: residues 1–18 comprising a mostly unstructured segment that is absolutely necessary for interactions with FliH, plus a globular segment comprising residues 20–91 that is conserved in FliI, type III ATPase and F1-ATPase sequences (supplemental Fig. S1). Our alignment and homology modeling are consistent with residues 20–91 of FliI forming a globular domain very similar in structure to the N-terminal $\beta$-barrel domain found in the F$_1$-ATPase catalytic subunits. Far-UV CD spectral analysis confirmed that residues 2–91 of *H. pylori* FliI adopt a mostly folded structure in solution that is absent of significant helical content, consistent with our structural model. Structural modeling further suggests...
FIGURE 6. Characterization of the FliI-(2–91)-FliH-(117–258) complex by gel filtration chromatography. Purified proteins (2 mg/ml FliI and FliH in PBS buffer) were mixed and incubated for 30 min at room temperature prior to injection on a Superdex HR-200 analytical gel filtration column equilibrated in PBS buffer. The flow rate was 0.5 ml/min, and protein was detected by UV absorbance at 280 nm. A, Superdex HR-200 elution profile of a roughly 1:1 mixture of FliI-(2–91) and FliH-(117–258). B, Superdex HR-200 elution profile of purified FliI-(2–91). C, Superdex 200 elution profile of the re-concentrated peak 1 from A. D, Coomassie Blue-stained 15% SDS-PAGE analysis of the peak fractions from A.

FIGURE 7. Characterization of a mutant FliI-(2–91)/FliH-(117–258) interaction and interaction of FliI-(19–91) with FliH-(117–258) by gel filtration chromatography. Buffer and incubation conditions were as in Fig. 6. A, Superdex HR-200 elution profile of a roughly 1:1 mixture of the L3A mutant of FliI-(2–91) and FliH-(117–258). B, Superdex HR-200 elution profile of purified FliI-(19–91). C, Superdex HR-200 elution profile of a roughly 1:1 mixture of FliI-(19–91) and FliH-(117–258).
residues 20–91 of FilI may participate in the recognition and binding of anionic phospholipids (Fig. 1). Published studies on the InvC type III ATPase demonstrate that amino acids within the InvC N-terminal domain interact with membrane lipids (21).

Analytical gel filtration studies of FilI-(2–91) and FilI-(19–91) demonstrate that these truncated domains form oligomers in solution, and our data are most consistent with these domains predominantly associating into trimers in vitro. Therefore, in vitro trimerization of the FilI N-domain may reflect the first steps in hexamerization of full-length FilI (23). It is noteworthy that the corresponding N-terminal domain in the F1-ATPase α- and β-subunits makes important subunit/subunit interactions and exhibits pseudo-hexameric symmetry in the functional F1-ATPase (αβ)3 heterotrimer (34–36).

The oligomerization properties of the H. pylori N-domain are in contrast with studies of full-length Salmonella FilI that indicate full-length FilI is largely a monomer in solution, except when ATP or anionic phospholipids are present (23, 27). Full-length H. pylori FilI is also monomeric in solution. Therefore, subunit/subunit interactions involving the truncated N-domain of FilI may behave differently in the context of the full-length FilI structure. In other words, the catalytic domain of FilI may impose structural constraints on subunit/subunit interactions involving the FilI N-domain, and these structural constraints may be sensitive to ATP binding in the catalytic domain.

Our work demonstrates that residues 1–18 of H. pylori FilI, although appearing unstructured in solution, are nevertheless absolutely required for interaction with FilH. That this segment likely forms an amphipathic α-helix upon interaction with FilH is supported by secondary structure predictions, helical wheel analysis, and mutagenesis results in combination with FilH binding studies. By using site-specific mutagenesis, we showed that three hydrophobic residues in the N-terminal FilI segment (leucine residues 3, 6, and 10) are absolutely required for the interaction with FilH. In contrast, most of the polar residues in this segment appear to have little effect on FilI-FilH complex formation. Hence, we predict that an FilI N-terminal amphipathic α-helix forms upon interaction with FilH and that a hydrophobic patch on this helix is critical for productive interactions with FilH. However, there is also a likely electrostatic component to the FilI/FilH interaction as mutation of Arg-9 to Ala did not noticeably affect the stability of the FilI-FilH complex, but the more drastic Arg-9 to Glu mutation significantly weakened complex formation.

Our studies provide significant new insight into the FilI/FilH interaction. First, although work on the Salmonella FilI/FilH interaction implicated the N terminus of FilI, the experiments either involved use of a drastic FilI R7C/L12P double mutant or used two products purified from limited proteolysis experiments (residues 7–456 and 26–456) (24, 26). Our work demonstrates exactly what residues are important for the interaction in H. pylori FilI (a hydrophobic patch of leucine residues) by using scanning alanine mutagenesis of carefully characterized truncated domains of FilI in combination with a number of truncated recombinant FilI proteins. Incidentally, our results predict that residues 5–12 (ILTRWLTLAL) (hydrophobic residues likely to interact with FilH indicated in bold) of Salmonella FilI likely make important contributions to the interaction with FilH (Fig. 1). We conclude that deletion of residues 1–7 of Salmonella FilI (24) likely impaired folding of residues 8–12 of that protein into a helical structure. This potentially also explains why the R7C/L12P double mutant does not interact with FilH (26). Furthermore, our work verifies and significantly extends preliminary yeast two-hybrid data reported for the H. pylori FilI/FilH interaction (12).

The FilI/FilH interaction was further analyzed by isolation and purification of the stable FilI-FilH complex by using gel filtration chromatography on mixtures of purified recombinant FilI-(2–91) and FilH-(117–258). The stoichiometry of the complex as determined by SDS-PAGE indicates an ~1:1 molar ratio of FilI-(2–91) and FilH-(117–258). The apparent molecular mass of this FilI-(2–91)-FilH-(117–258) complex is ~1.3 × 106 Da, indicating the subunit stoichiometry in the complex is different from the (FilH)2-FilI stoichiometry reported for the full-length Salmonella FilI-FilH complex (24). Hence it appears that the N-domain of H. pylori FilI may also behave differently upon interacting with FilH. The functional significance of the FilI-(2–91)-FilH-(117–258) complex is presently unclear.

Data presented in this paper strongly support the contention that residues 1–14 of FilI form an amphipathic α-helix on interaction with FilH. Most intriguingly, the related F1-ATPase α-subunit has been shown to contain an amphipathic α-helix at its N terminus, just preceding the β-barrel domain (supplemental Fig. S1) (42). Furthermore, it is compelling that the F1-ATPase α-subunit uses the hydrophobic surface of this α-helix to facilitate protein/protein interactions with the F1-ATPase δ-subunit (42). With FilI, we have shown that hydrophobic residues on this presumed N-terminal helix mediate interactions with FilH. The F1-ATPase δ-subunit, in combination with the F0,F1-ATPase b-subunit, is known to function as the stator of the F0,F1-ATPase rotary motor, forming an elongated “outer stalk” and preventing unwanted rotation of the F1 catalytic subunits relative to the rotation of the torque-generating γ-subunit (35, 42). We note that the b-subunit of F1-ATPase forms a highly elongated dimeric structure, again reminiscent of the elongated dimeric solution structure of Salmonella and presumably H. pylori FilH. The actual biological function of FilH in flagellar protein export is not known, although in vitro it acts as an inhibitor of FilI catalytic activity (26) and is also known to interact with membrane-embedded components of the flagellar export apparatus and anionic phospholipids (22, 25). Consistent with our observation of similarity in solution properties between FilI and the F0,F1-ATPase b-subunit, other authors have previously noted weak sequence similarity between a segment of the F0,F1 b-subunit and FilH, suggesting the possibility of an evolutionary relationship between these proteins (43, 44). We suggest that the FilI/FilH interactions demonstrated in this report are analogous to the observed interactions between F1-α- and F1-δ-subunits in the F0,F1-ATPase (42). This implies that the C-domain of FilH (like the F1-δ-subunit) would have a mostly globular structure responsible for interactions with FilI. Bioinformatic data indeed suggest that the C-domain of FilH adopts a globular structure.6

Previously published data support the idea that FilI functions as a hexameric ring structure (23). The nature of the FilI/FilH interaction and its uncanny similarity to the interaction between an amphipathic helix on the F1-ATPase α-subunit and a globular domain on the F1-ATPase δ-subunit lead us to suggest that FilH could function as the FilI stator. The overall structural features of FilH, a mostly helical N terminus and a globular C-domain together making up an elongated structure, are reminiscent of structural features of the F1-ATPase stator. Structural studies of H. pylori FilI and FilH are in progress in our laboratories to further elucidate the structure/function relationships of these proteins.

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