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Host cell binding of the flagellar tip protein of *Campylobacter jejuni*

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Abstract

Flagella are nanofibers that drive bacterial movement. The filaments are generally composed of thousands of tightly packed flagellin subunits with a terminal cap protein, named FliD. Here, we report that the FliD protein of the bacterial pathogen *Campylobacter jejuni* binds to host cells. Live-cell imaging and confocal microscopy showed initial contact of the bacteria with epithelial cells via the flagella tip. Recombinant FliD protein bound to the surface of intestinal epithelial cells in a dose-dependent fashion. Search for the FliD binding site on the host cell using cells with defined glycosylation defects indicated glycosaminoglycans as a putative target. Heparinase treatment of wild type cells and an excess of soluble heparin abolished FliD binding. Binding assays showed direct and specific binding of FliD to heparin. Addition of an excess of purified FliD or heparin reduced the attachment of viable *C. jejuni* to the host cells. The host cell binding domain of FliD was mapped to the central region of the protein. Overall, our results indicate that the *C. jejuni* flagellar tip protein FliD acts as an attachment factor that interacts with cell surface heparan sulfate glycosaminoglycan receptors.

KEYWORDS

Campylobacter, epithelial cells, flagella, FliD, heparan sulfate, receptor

1 | INTRODUCTION

Bacterial pathogens need to interact with eukaryotic cells to establish an infection. Initial host cell contact is often conferred by hair-like appendages (termed pili or fimbriae) that protrude from the bacteria and that can bridge the electrostatic barrier with the host cell surface. The pilus filaments consist of polymers of thousands of protein subunits (pilins) and usually carry one or more tip proteins that bind to host cell surface receptors (Kline, Fälker, Dahlberg, Normark, & Henriques-Normark, 2009). Another bacterial structure that extends from the bacterial surface and often contributes to bacterial pathogenesis is the flagellum. Like a pilus, the flagellum is composed of thousands of subunits (flagellins) that are assembled into a hollow fiber that is capped at its distal end by the flagellar cap protein, termed FliD (or HAP2). The flagellar fiber is anchored via a short hook filament structure to the flagellar motor complex in the bacterial membrane. This nanomachine drives the rotation of the flagella enabling movement of the bacteria. Besides its role in bacterial motility, the flagellar apparatus may act as a secretion machinery that exports proteins into the environment or the host cell cytosol (Neal-McKinney & Konkel, 2012; Young, Schmiel, & Miller, 1999). This function is consistent with the ancestral resemblance between the bacterial flagellar apparatus and type III secretion systems (T3SSs) present in many Gram-negative bacterial pathogens (Hueck, 1998). T3SSs form a needle-like structure that may inject effector molecules into eukaryotic cells to modulate host cell function (Cornelis, 2006; Galán & Wolf-Watz, 2006).

The bacterial pathogen *Campylobacter jejuni* is the most frequent cause of bacterial foodborne disease (European Food Safety Authority and European Centre for Disease Prevention and Control, 2015). In the natural infection, *C. jejuni* causes severe diarrheal infection and can be observed in strong association with and inside intestinal epithelial cells (van Spreeuwel et al., 1985). The molecular pathogenesis of the infection is still largely unknown. *C. jejuni* lacks pili for initial adherence to host cells and does not show T3SS-dependent bacterial invasion of

host cells. However, the pathogen does carry a single flagellum at one or both of its polar ends (for review, see van Putten, Alphen, Wösten, & Zoete, 2009). The major building blocks of the *C. jejuni* flagellum are the flagellin subunits FlaA and FlaB. The filament is supposedly capped by a FliD protein complex as reported for other bacterial species (Yonekura et al., 2000). Typically, the *C. jejuni* flagellum is heavily glycosylated and its motor appears larger and more complex than in most enteric bacteria (Beeby, 2015). These traits likely enable *C. jejuni* to penetrate the viscous mucous barrier and to colonize the intestinal crypts (Lee, O'rourke, & Barrington, 1986). Infection of human volunteers and animals with a mixture of motile and immotile *C. jejuni* showed only recovery of flagellated bacteria, indicating that the flagella are crucial for colonization (Aguero-Rosenfeld, Yang, & Nachamkin, 1990; Black, Levine, Clements, Hughes, & Blaser, 1988).

A unique trait of *C. jejuni* appears to be the secretion of effector molecules through the flagellar apparatus rather than via a typical T3SS (Konkel, Kim, Rivera-Amill, & Garvis, 1999; Konkel et al., 2004; Neal-McKinney & Konkel, 2012). Some of those exported proteins, collectively called *Campylobacter* invasion antigens, modulate host cell biology (Krause-Gruszczynska et al., 2007; Samuelson et al., 2013). How the proteins that are secreted via the flagellar apparatus of *C. jejuni* are delivered into the cells is still unresolved. Several other *C. jejuni* proteins have been proposed to contribute to early stages of cellular infection including the aspartate/glutamate binding protein Peb1A (Leon-Kempis, Guccione, Mulholland, Williamson, & Kelly, 2006; Pei et al., 1998), the surface exposed lipoprotein JlpA (Jin et al., 2003), the fibronectin binding protein CadF (Konkel et al., 1997; Krause-Gruszczynska et al., 2007; Monteville, Yoon, & Konkel, 2003), the major outer membrane protein MOMP (Moser, Schroeder, & Salnikow, 1997), the FlpA protein (Flanagan, Neal-McKinney, Dhillon, Miller, & Konkel, 2009), and the autotransporter protein CapA (Ashgar et al., 2007). Which factor(s) confer(s) the initial contact with the host is still unknown.

Considering the central role of the flagellar apparatus in different steps in *C. jejuni* pathogenesis and microscopy observations on infected host cells, we hypothesized that the *C. jejuni* flagella and, in particular, the FliD tip protein may directly interact with the host cells. Here, we provide evidence that the *C. jejuni* flagellar tip protein FliD binds to host cells through specific interaction of the central region of the FliD protein with cell surface heparan sulfate glycosaminoglycan receptors.

2 | RESULTS

2.1 | Flagella-dependent anchoring of *C. jejuni* to host cells

Live imaging of cultured epithelial cells inoculated with *C. jejuni* strain 81116 and confocal fluorescent microscopy on cells with mCherry-expressing bacteria and differentially stained flagella repeatedly showed low numbers of free-floating bacteria that seemed to be tethered to the cells via the tip of the flagella (Figure 1A and 1B and S1). These observations led us to hypothesize that the flagellar cap protein FliD of *C. jejuni* may interact with host cells. To test this scenario, we first constructed a FliD mutant strain by allelic replacement of the *C. jejuni fliD* gene with an antibiotic resistance cassette. As FliD is important for flagella assembly (Ikeda, Yamaguchi, & Hotani, 1993) and thus bacterial motility, FliD was inactivated in the wild type strain 81116 as well as its immotile derivatives 81116Δ*flaAB* that is unable to produce the flagellin subunits, and 81116Δ*motAB* that lacks the *motAB* genes essential for the rotation of the flagella. Motility testing of the strains confirmed that inactivation of *flaAB*, *motAB*, and/or *fliD* resulted in immotile bacterial phenotypes (Figure 1C and 1D). Because FliD forms the flagellar cap protein complex important for filament growth and stabilization (Diószeghy, Závodszky, Namba, & Vonderviszt, 2004; Vonderviszt et al., 1998), we also verified flagellin expression in the various strains. The mutants 81116Δ*fliD* and 81116Δ*motAB*Δ*fliD* still expressed bacteria-associated flagellins, although levels were slightly reduced compared to the parent strain (Figure 1E). Infection of epithelial cells with strain 81116Δ*motAB*Δ*fliD* and its parent 81116Δ*motAB* showed 70–95% reduction in *C. jejuni* adhesion for the FliD-deficient strain as determined by microscopy (Figure 1F) and viability counting of cell-associated bacteria (Figure 1G). Together, these data support the notion that FliD is important for the initial interaction of *C. jejuni* with epithelial cells.

2.2 | Cloning and purification of the *C. jejuni* flagellar tip protein FliD

In order to better assess the putative cell-binding properties of FliD, the gene was cloned with a C-terminal Tobacco Etch Virus (TEV) cleavage site followed by a His-tag in the expression vector PCP1. After removal of the His-tag with TEV protease, the purified recombinant FliD migrated on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as a single band with the expected molecular mass of approximately 70 kDa (Figure 2A and 2B). Immunization of rabbits with the recombinant protein yielded antibodies that specifically recognized the 70 kDa band in the Western blot (Figure 2C). Reactivity with this protein was observed for all tested *C. jejuni* wild-type strains (11168, 81116, 108, 81–176) but not for the FliD-negative strains (81116Δ*fliD* and 81116Δ*motAB*Δ*fliD*; Figure 2D), indicating that the antisera recognized *C. jejuni* FliD of multiple strains consistent with the conserved nature of the protein.

2.3 | Binding of recombinant FliD to mucosal epithelial cells

The cell-binding properties of the recombinant FliD protein were investigated in a cell-based enzyme-linked immunosorbent assay (ELISA) that involved incubation of purified protein with cultured eukaryotic cells followed by quantification of FliD binding using the generated anti-FliD antibodies in combination with a horseradish peroxidase (HRP)-labeled conjugate. Epithelial cells were fixed to prevent detachment of cells and potential internalization of FliD during the assay. This approach demonstrated binding of FliD to the epithelial cells in a dose-dependent fashion reaching a maximum of binding at a protein concentration of 10 μg/ml (Figure 3A). Fluorescence microscopy of nonfixed FliD-exposed (Figure 3B upper panel) and control cells (Figure 3B lower panel) using a red-fluorescent-labeled conjugate to visualize the protein, confirmed the presence of discrete clusters of

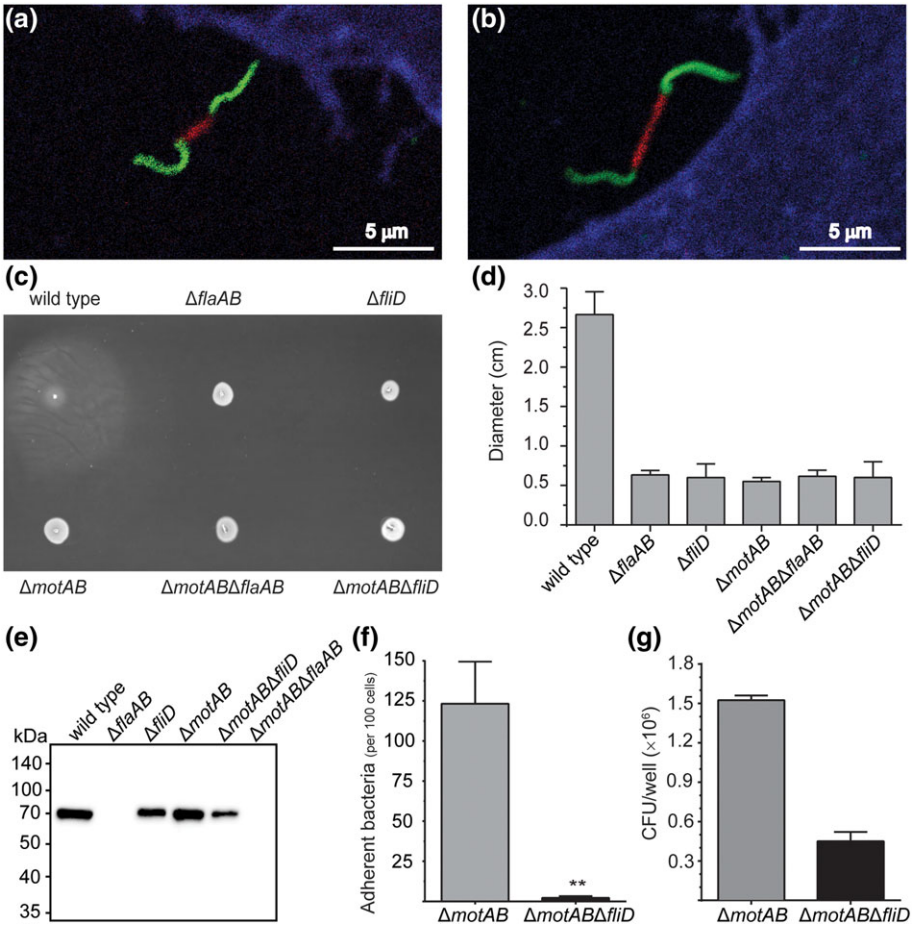

FIGURE 1 Flagella tip mediated attachment of *C. jejuni* to epithelial cells. (a) and (b) Immunofluorescence microscopy on *C. jejuni* 81116 wild-type infected Chang epithelial cells. After 2-hr infection, cells were washed and fixed. The cell surface was stained with WGA-Alexa Fluor 633 (blue). The flagella of mCherry-positive bacteria (red) were visualized using anti-*C. jejuni* antibodies, followed by goat-anti-rabbit-IgG Alexa Fluor 488 (green). (c) and (d) Effect of inactivation of *fliD* and *flaAB* on the motility of strain 81116 and its *motAB* derivatives in soft agar plates (c), as quantified by growth zone diameter (d). (e) Flagellin levels in bacterial pellets of strain 81116 and the indicated mutants as determined by Western blot. Bacterial lysates were separated by SDS-PAGE, blotted, and probed with anti-flagellin antiserum followed by goat-anti-rabbit-IgG-HRP. (f) FliD-dependent attachment of *C. jejuni* to epithelial cells as determined by microscopy. Cells were incubated with the indicated strains for 2 hr, fixed, and stained with 0.01% crystal violet. Bacterial adhesion was quantified by light microscopy. Each bar represents the mean ± SEM of three experiments. Data were analyzed by Student's *t* test (**p = .01). (g) FliD-dependent attachment of *C. jejuni* to epithelial cells as determined by viability counting. After 2 hr of infection, the number of attached bacteria as recovered as colony forming units (CFU) on blood agar plates was counted. Each bar represents the mean ± SD of duplicate values of one representative of three experiments. SDS-PAGE, SDS-polyacrylamide gel electrophoresis

FliD at the apical surface of the cells. Similar assays with the intestinal cell lines HRT-18 and HT-29 cells revealed that FliD also bound to these cells in a dose-dependent manner (Figure 3C). Immunofluorescence microscopy confirmed binding of FliD to both types of intestinal cells, with a trend towards increased binding to HT-29 cells (Figure 3D and 3E upper panels) probably due to a different differentiation status of the cell lines (Molist et al., 1998). The staining was not observed for non-FliD exposed control HRT-18 and HT-29 cells (Figure 3D and 3E lower panels).

2.4 | Characterization of the FliD binding site on the eukaryotic cells

As bacterial pathogens often exploit cell surface glycoproteins as host cell receptors (Lehmann, Tiralongo, & Tiralongo, 2006), we first tested the binding of FliD to Chinese hamster ovary (CHO) cells with defects in their N- or O-glycosylation machinery using the cell-based ELISA. FliD binding to CHO-lec2 and CHO-lec8 cells that lack terminal sialic acids and terminal galactose residues on their respective sugar chains (Patnaik & Stanley, 2006), was unaltered compared to the binding of FliD to wild-type CHO-K1 cells (Figure 4A). However, FliD bound significantly less to CHO-745 cells defective in glycosaminoglycan (GAG) synthesis (Esko & Stanley, 2009; Figure 4A). In line with this finding, preincubation of cells with sodium chlorate, which inhibits sulfation of proteins and carbohydrates including glycosaminoglycans (Baeuerle & Huttner, 1986), reduced FliD binding significantly (Figure 4B).

To investigate the class of GAGs responsible for the binding of FliD, we incubated the recombinant protein with various types of GAGs prior to addition to the cells. Effective dose-dependent inhibition of binding was obtained with heparin in the concentration range between 1 and 100 ng/ml (Figure 4C). The addition of

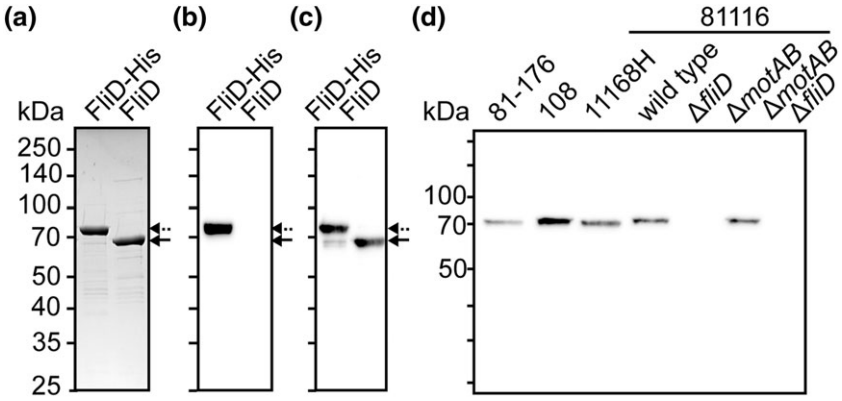

FIGURE 2 Isolation of recombinant FliD and reactivity of the generated antiserum. (a) SDS-PAGE of purified recombinant *C. jejuni* FliD protein before (dotted arrow) and after (solid arrow) removal of the His-tag with TEV protease. Proteins were stained with PageBlue. (b) and (c) Western blot of the same samples showing the reactivity of the FliD protein with (b) antibodies directed against the His-tag and (c) the raised FliD antisera. (d) Western blot showing the reactivity of the FliD antiserum with FliD in lysates of the *C. jejuni* wild-type strains (81–176, 108, 11168H, and 81116) and the indicated mutant strains. As expected, the FliD-negative strains 81116Δ*fliD* and 81116Δ*motAB*Δ*fliD* lacked FliD reactivity. SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TEV, tobacco etch virus

hyaluronic acid, chondroitin sulfate A, or dermatan sulfate did not reduce FliD binding, even at concentrations of up to 1,000 ng/ml (Figure 4C).

To confirm heparan sulfate containing molecules as putative cell surface binding partner of FliD, we treated epithelial cells with the enzyme Heparinase-I, which removes heparan sulfate from the cells surface (van Putten & Paul, 1995). Heparinase treatment reduced the binding of FliD to the cells in an enzyme-activity-dependent fashion, whereas treatment of the cells with chondroitinase ABC had no effect on the binding of FliD (Figure 4D).

We also cloned and expressed the FliD protein of the frequently studied isolate *C. jejuni* strain 11168 (94% amino acid sequence similarity). As expected, this protein showed similar host cell binding characteristics as noted for FliD protein of strain 81116 (Figure 4E). Overall, our findings strongly suggest that *C. jejuni* FliD binds to heparan sulfate moieties at the epithelial cell surface.

2.5 | Direct interaction of FliD with heparin

To demonstrate direct interaction of FliD with heparan sulfate, we coated recombinant FliD in an ELISA setup and binding of biotinylated heparin was measured using HRP-streptavidin as conjugate. In line with the results above, heparin bound to FliD in a dose-dependent manner (Figure 5A). The specificity of the interaction was demonstrated by the inability of chondroitin sulfate A, dermatan sulfate, or hyaluronic acidN to outcompete the FliD binding of biotinylated heparin, whereas heparin strongly reduced the binding of the biotinylated molecule (Figure 5B).

2.6 | Native *C. jejuni* FliD binds to heparan sulfate glycan receptors

To ensure that the observed FliD binding properties obtained with recombinant protein mimicked those of natural *C. jejuni* FliD, we tested the binding characteristics of native FliD protein. The native protein is present in high amounts in the culture supernatant of the *C. jejuni* 81116Δ*flgKM* mutant, which has a defect in flagella filament formation (Bleumink-Pluym, Verschoor, Gaastra, Zeijst, & Fry, 1999; Figure 6A). Incubation of epithelial cells with concentrated culture supernatant of this mutant resulted in a dose-dependent binding of FliD to the cells (Figure 6B), as was noted earlier for recombinant FliD. To ascertain that the observed binding required cell surface GAGs, we incubated FliD containing supernatant with different concentrations of heparin or dermatan sulfate prior to addition to cells. Consistently, heparin inhibited the binding of native FliD to the cells at the low dose of 10–100 ng/ml of heparin, whereas dermatan sulfate had no effect (Figure 6C).

2.7 | FliD and heparin reduce adhesion of *C. jejuni* to host cells

To assess whether the binding of FliD to cell surface heparan sulfate receptors could be exploited to inhibit *C. jejuni* adhesion to host cells, we investigated the role of soluble FliD protein and heparin in an infection setting. Epithelial cells were incubated with recombinant FliD (25 μg/well) and subsequently infected with *C. jejuni* strain 81116Δ*motAB*, which displays higher adhesive potential than the wild type probably due to its defect in bacterial motility (Mertins, Allan, Townsend, Köster, & Potter, 2013). Quantification of adherent bacteria showed that preincubation of the cells with FliD reduced *C. jejuni* adhesion by approximately 65% (Figure 6D). A similar strong reduction in *C. jejuni* attachment was observed when the bacteria were preincubated with heparin prior to their addition to the cells (Figure 6D). These results strongly suggest that *C. jejuni* FliD is a major attachment factor and that the *C. jejuni* adhesin to host cells can be inhibited by the use of competing purified FliD protein as well as a soluble receptor analogue.

2.8 | Mapping of the host cell binding region in FliD

In the absence of any three-dimensional structural information of the *C. jejuni* FliD protein, we used the protein information of the FliD

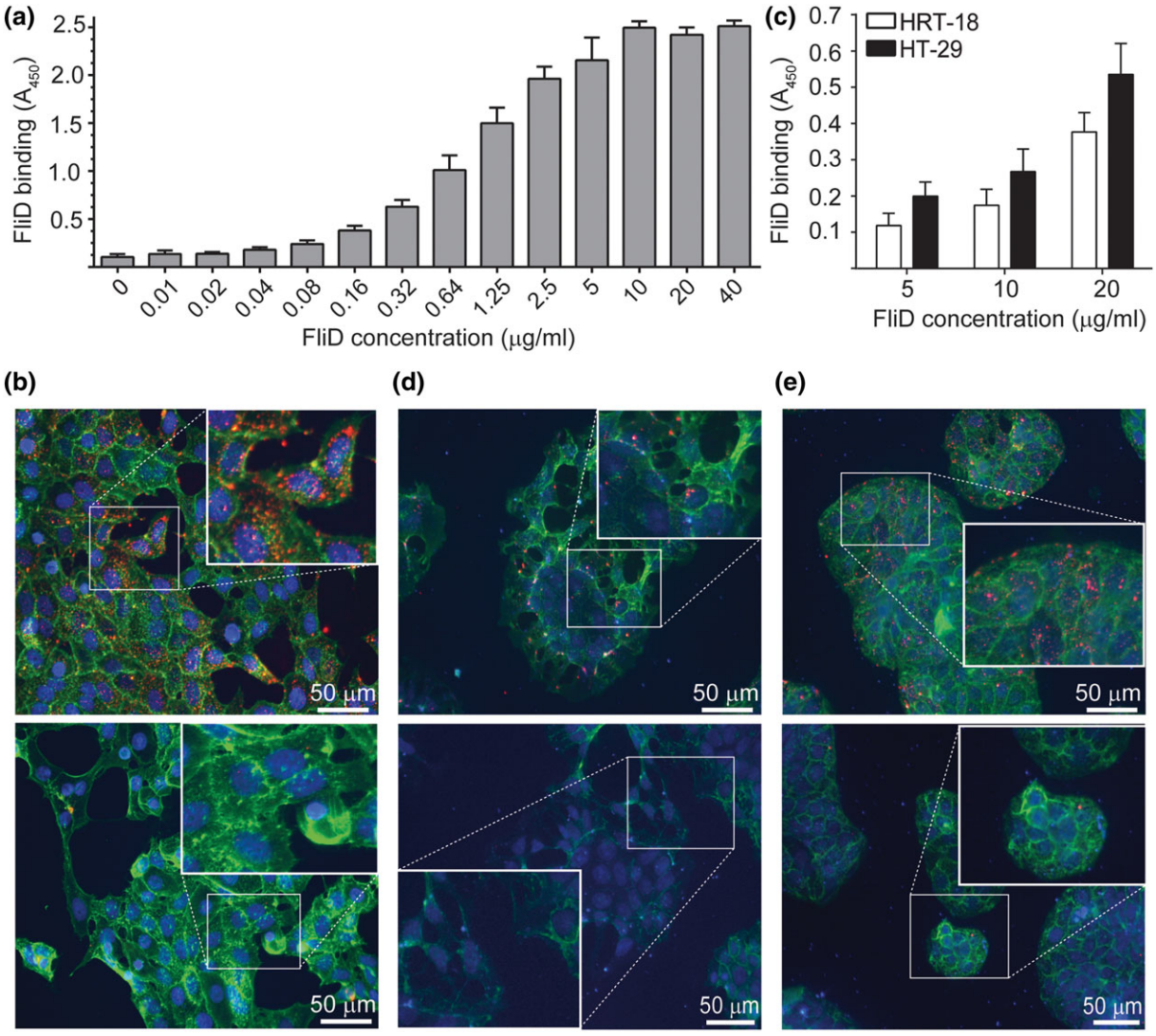

FIGURE 3 FliD binding to different types of epithelial cells. (a) Cell-based ELISA results showing the binding of recombinant *C. jejuni* FliD protein to fixed Chang epithelial cells after incubation (1 hr, 20 °C) with the indicated protein concentrations. Binding was quantified using anti-FliD antibodies in combination with HRP-conjugated secondary antibodies. Results represent the mean ± SEM of three experiments. (b) Immunofluorescence microscopy on unfixed Chang cells incubated with (upper panel) or without (lower panel) FliD protein (2 μg/well, 1 hr). Nuclei were stained with DAPI (blue). The cell surface was stained with WGA-Alexa 488 (green). FliD was detected using anti-FliD antibodies followed by goat anti-rabbit-IgG Alexa 568 (red). The inserts show a magnified view of the marked areas, demonstrating clusters of FliD bound to the cell surface. (c) Binding of the indicated concentrations of FliD protein to HRT-18 and HT-29 intestinal epithelial cells as determined by cell-based ELISA. Each bar represents the mean ± SEM of three experiments after background subtraction. (d) and (e) Immunofluorescence microscopy on (d) HRT-18 and (e) HT-29 intestinal epithelial cells incubated with (upper panel) or without (lower panel) FliD protein (2 μg/well, 1 hr). Staining was performed as described in Figure 3b. ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase

protein of *Salmonella* Typhimurium as a starting point to map the host cell binding domain in FliD. The FliD protein of *S.* Typhimurium forms a pentameric complex with a central pentagonal plate and five leg domains that are buried within the flagellar structure. The terminal regions of the different FliD proteins in the complex likely form a coiled-coil structure that interacts with the flagellin subunits at the filament end (Vonderviszt et al., 1998; Yonekura et al., 2000). On the basis of this model, we first constructed a *C. jejuni* FliD protein that lacked the first N-terminal 70 amino acids of the protein and the last 87 residues at the C-terminal end (Figure 7A). The purified protein (named FliDΔNC) migrated on SDS-PAGE with the expected molecular mass of 60 kDa (Figure 7B). Cell binding assays with the recombinant FliDΔNC protein using the polyclonal anti-FliD antiserum as a probe showed similar cell binding activity as observed for the full length protein (Figure 7C), indicating that the N- and C-terminal regions are not required for cell binding.

To further map the potential binding region, we fragmented the FliDΔNC protein by cloning three DNA segments, named A, B, and C (Figure 7A). In order to ensure equal detection of the different protein fragments in the binding assay, we cloned the constructs with a C--terminal sortase-binding motif, which enabled site-specific biotinylation and detection with streptavidin-HRP (Popp, Antos, Grotenbreg, Spooner, & Ploegh, 2007). Expression and purification of the corresponding proteins resulted in three protein fragments with apparent molecular masses of 29, 35, and 32 kDa, respectively (Figure 7D), which after the sortase labeling procedure migrated on SDS-PAGE

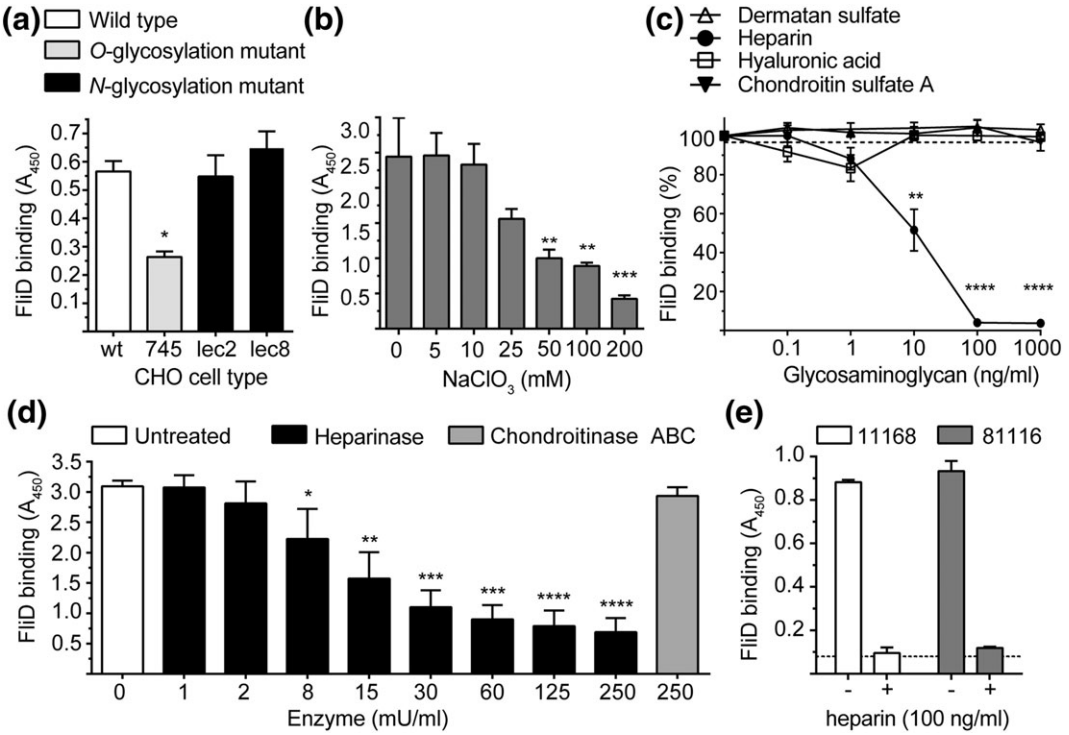

FIGURE 4 Binding of FliD to heparan sulfate moieties on the host cell surface. (a) FliD-specific cell-based ELISA showing the binding (1 hr) of FliD protein (20 μg/ml) to CHO-K1 wild type cells, the *N*-glycosylation mutants CHO-lec2 and CHO-lec8 cells, and the glycosaminoglycan deficient CHO-745 cells. Results are the mean ± SEM of three independent experiments. Statistical significance was calculated using one-way ANOVA (**p* < .05). (b) FliD-binding cell-based ELISA showing the binding (1 hr) of FliD to Chang cells pretreated (16 hr) with the indicated concentrations of the sulfation inhibitor sodium chlorate ($NaClO_3$). Results are the mean ± SEM of three independent experiments. Statistical significance was calculated using one-way ANOVA (***p* < .01 and ****p* < .001). (c) Competitive effect of the indicated types of glycosaminoglycans on FliD binding to Chang cells. FliD protein was preincubated with the indicated concentrations of different compounds and then subjected to the cell-based ELISA procedure. Results are the mean ± SEM of three independent experiments. Statistical significance was calculated using two-way ANOVA (***p* < .01 and *****p* < .0001). (d) FliD-specific cell binding after treatment (1 hr, 37 °C) of Chang cells with the indicated concentrations of Heparinase or Chondroitinase ABC. The binding assay was performed as described under Figure 4a. Results are the mean ± SD of three independent experiments. Statistical significance was calculated using one-way ANOVA (***p* < .01, ****p* < .001, and *****p* < .0001). (e) FliD protein of *C. jejuni* strains 81116 and 11168 was incubated with heparin prior addition to Chang cells as described under Figure 4c. Results represent the mean ± SD of duplicates of one representative of three independent experiments. ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay

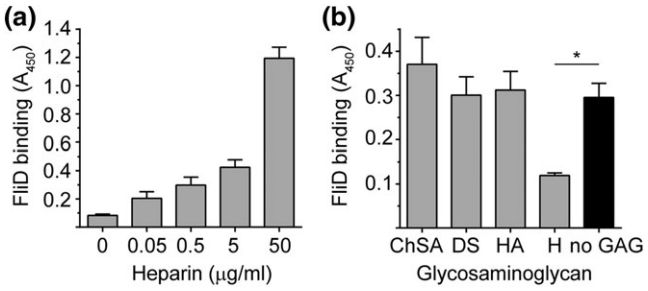

FIGURE 5 Direct and specific interaction of FliD with heparin. (a) Binding assay showing the dose-dependent binding of the indicated concentrations of biotinylated heparin to recombinant *C. jejuni* FliD coated onto ELISA plates. Heparin binding was measured using streptavidin-HRP. Results represent the mean ± SEM of three independent experiments. (b) Binding assay showing the competitive effect of unbiotinylated chondroitin sulfate A (ChSA), dermatan sulfate (DS), hyaluronic acid (HA), and heparin (H) on the binding of biotinylated heparin to immobilized *C. jejuni* FliD. Biotinylated heparin binding was detected with streptavidin-HRP. Results represent the mean ± SEM of three independent experiments. Only an excess of heparin was able to compete with biotinylated for FliD binding as determined by Student's *t* test (**p* < .05). ELISA, enzyme-linked immunosorbent assay;HRP, horseradish peroxidase

with apparent molecular masses of approximately 13, 24, and 14 kDa, respectively (Figure 7D). Western blotting confirmed the successful biotinylation of the protein fragments (Figure 7E). Incubation of the protein fragments A, B, and C with cells showed that only fragment B displayed strong cell binding activity (Figure 7F), suggesting that the cell binding site encompasses the central part (amino acids 209–418) of the FliD protein.

3 | DISCUSSION

Bacterial movement and firm attachment to host cells are important virulence traits of many pathogens. Here, we provide evidence that the flagellar tip protein FliD of *C. jejuni* is not only required for bacterial motility but also acts as an early attachment factor. FliD-deficient strains displayed strongly reduced bacterial adhesion to epithelial cells and recombinant FliD as well as native soluble FliD bound to the surface of cells. We identified heparan sulfate glycosaminoglycans as the binding partner of FliD on the host cells and found that both soluble FliD and the receptor analogue heparin can inhibit *C. jejuni* attachment to the cells. The dual function of FliD as capping protein

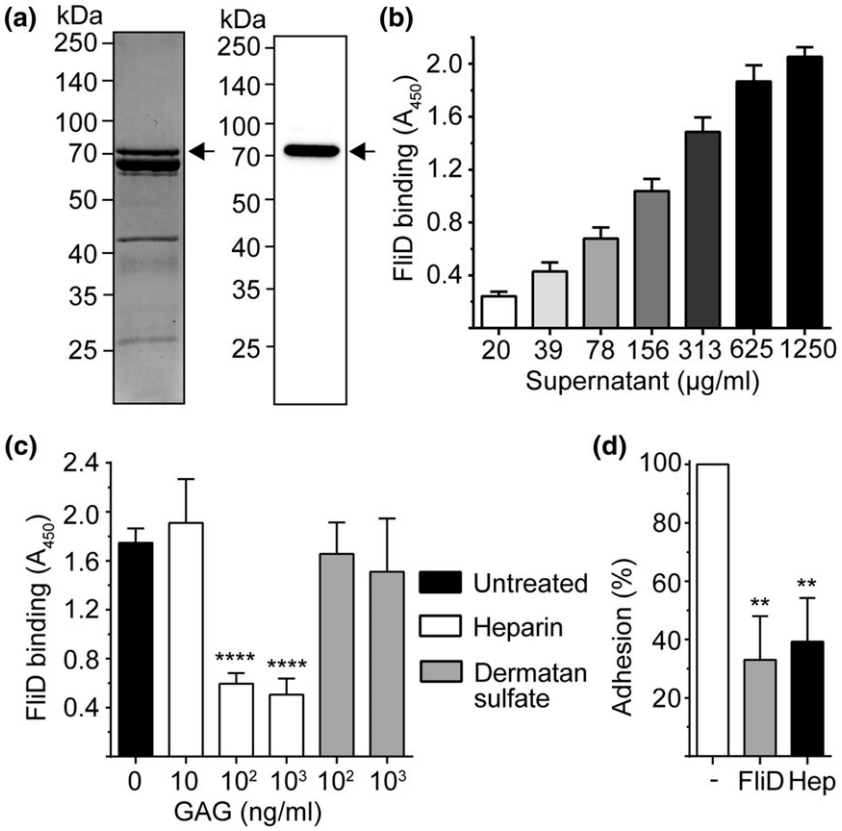

FIGURE 6 Binding of *C. jejuni*-derived FliD and intact *C. jejuni* to glycosaminoglycans and its inhibition by soluble FliD protein and heparin. (a) SDS-PAGE (left panel) and Western blot (right panel) illustrating the presence of FliD in the culture supernatant of strain 81116Δ*flgKM*. Proteins were stained with PageBlue. The blot was probed with the FliD antiserum and HRP-conjugated goat-anti-rabbit IgG. (b) Cell-based ELISA demonstrating the binding (1 hr, 20 °C) of *C. jejuni*-derived FliD to Chang cells. Binding was measured following the addition of increasing concentrations of FliD containing bacterial supernatant to the cells and detected using FliD antiserum and HRP-conjugated goat anti-rabbit IgG. (c) Cell-based ELISA showing the effect of the indicated concentrations of heparin and dermatan sulfate on the binding of FliD present in *C. jejuni* culture supernatant (625 µg protein/ml, black bar) to Chang cells. Binding of FliD was detected using FliD antiserum and HRP-conjugated goat anti-rabbit IgG. Data represent the mean ± SEM of three independent experiments. Only an excess of heparin was able to significantly inhibit the binding of *C. jejuni* FliD as determined by one-way ANOVA in comparison to the untreated control (****$p < .0001$). (d) Binding of viable *C. jejuni* (strain 81116Δ*motAB*) to Chang cells after preincubation (1 hr) of the cells with buffer (white bar) or recombinant FliD (25 µg, grey bar), or incubation of bacteria with soluble heparin (0.5 mg, black bar). Bacterial attachment was determined by counting the number of attached bacteria for at least 300 cells and expressed as relative adhesion compared to the nontreated control. Results are the mean ± SD of three experiments. Statistical significance was calculated using Student's *t* test in comparison to the untreated control (**$p < .01$). ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

important for flagella assembly and as flagellar tip attachment factor underscores the proposed key role of the flagella in the natural *C. jejuni* infection (Black et al., 1988).

The hypothesis that *C. jejuni* FliD may act as an attachment factor was based on live imaging and fluorescence microscopy on infected epithelial cells that showed the marked presence of bacteria that seemed anchored to the host cells via their flagellar tip. This feature did not require flagellar rotation as bacterial attachment was even stronger in a *motAB* mutant that lacks the proteins that drive the rotation of the flagella (Mertins et al., 2013). Genetic inactivation of FliD largely abolished this initial *C. jejuni* attachment. It remains a major challenge to investigate FliD-mediated attachment *in vivo* due to the lack of appropriated animal models and the necessity of motility for efficient *C. jejuni* colonization (Mertins et al., 2013).

As FliD is essential for the polymerization of the flagellin subunits into the filament, the lack of bacterial adhesion to the host cells may theoretically reflect the absence of flagellins rather than FliD per se. This was excluded after investigation of the binding properties of purified recombinant FliD. This demonstrated direct and dose-dependent binding of *C. jejuni* FliD to the epithelial cells.

A role of flagella in bacterial attachment to host cells has previously been suggested for various bacterial species (Haiko & Westerlund-Wikström, 2013) including *C. jejuni* (Konkel et al., 2004; McSweegan & Walker, 1986), *Pseudomonas aeruginosa* (Bucior, Pielage, & Engel, 2012), *Clostridium difficile* (Baban et al., 2013), *Bordetella pertussis* (Savelkoul et al., 1996) and commensal species (Troge et al., 2012). In most cases, this concept was based on investigation of the adhesive potential of the entire flagella (comprising both flagellins and flagellar cap) and hence the specific role of FliD was not further clarified. For pathogenic *E. coli*, both flagella and isolated flagellins have been proposed as adhesion factors (Erdem, Avelino, Xicohtencatl-Cortes, & Girón, 2007; Girón, Torres, Freer, & Kaper, 2002). This adhesion may be mediated by the soluble EtpA adhesin that anchors to the distal end of the *E. coli* flagella probably after loss

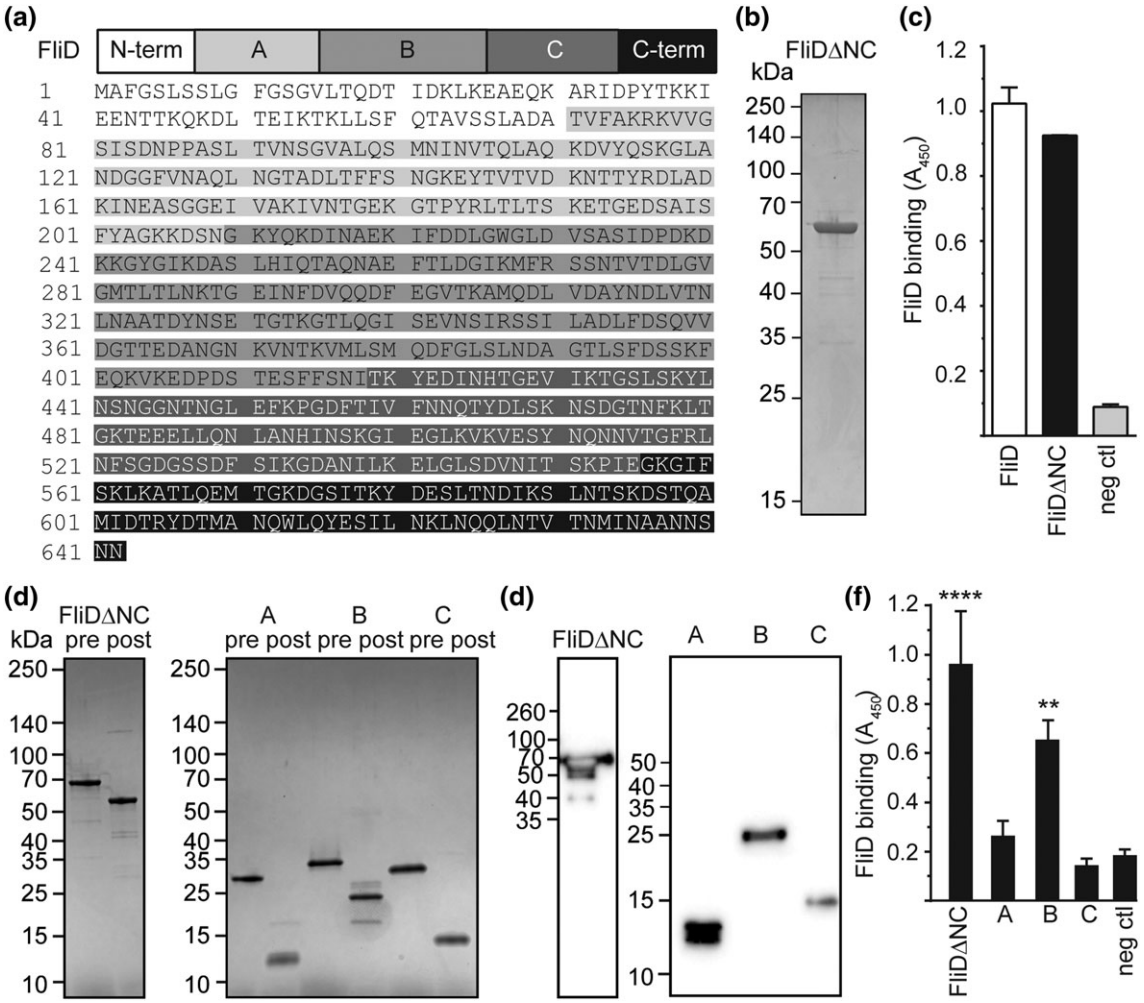

FIGURE 7 The central region of FliD is important for cell binding. (a) Graphical representation of the FliD protein sequence of strain 11168 indicating the N- and C-terminal domains and the cloned FliD fragments (A–C). The grey shades of the FliD fragments correspond to the highlighted protein sequences, respectively. (b) SDS-PAGE demonstrating the migration of pure FliD protein lacking its N- and C-terminal ends (FliDΔNC). The protein was visualized with PageBlue. (c) Cell-based ELISA results after the addition of full length recombinant *C. jejuni* FliD (40 μg/ml), FliDΔNC or buffer only (neg ctl) to fixed Chang epithelial cells. Binding was quantified using anti-FliD antibodies in combination with HRP-conjugated secondary antibodies. Results represent the mean ± SD of duplicates of one representative of three independent experiments. (d) SDS-PAGE of isolated FliDΔNC protein and the FliD protein fragments A, B, and C before and after the sortase reaction. Proteins were stained with PageBlue. (e) Western blot of the biotinylated FliDΔNC protein and the FliD protein fragments A, B, and C probed with streptavidin-HRP. (f) Cell-based ELISA showing the binding of the biotinylated FliDΔNC protein and the FliD protein fragments A, B, and C (75 pmol) to Chang cells. Binding was detected using streptavidin-HRP. Incubation of the cells with buffer only served as control (neg ctl). Results are the mean ± SD of three experiments. Statistical significance was calculated using one-way ANOVA in comparison to the negative control (**$p < .01$ and ****$p < .0001$). ANOVA, analysis of variance; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

of the FliD cap complex (Roy et al., 2009). To our knowledge, the only identified FliD proteins with adhesive properties thus far are the FliD of *P. aeruginosa* (Arora, Ritchings, Almira, Lory, & Ramphal, 1998) and of *C. difficile* (Tasteyre et al., 2001). *P. aeruginosa* FliD confers binding to Lewis(x) or sialyl-Lewis(x) determinants present on the surface on cells and on mucins (Scharfman et al., 2001). For *C. difficile* FliD, no cellular binding partner has thus far been identified.

Our successful search for the *C. jejuni* FliD receptor(s) on the host cells was important to validate the specificity of the binding of *C. jejuni* FliD to the cells. We identified heparan sulfate glycosaminoglycans as main binding partner of FliD. This discovery is based on the reduced binding of FliD to glycosaminoglycan-deficient host cells, the inhibition of FliD binding by soluble heparin but not by other glycosaminoglycans, the reduced FliD binding after treatment of the cells with heparinase-I, and the direct binding of FliD to heparin. The reduced FliD binding after treatment of the cells with sodium chlorate, which prevents the sulfation of glycoproteins and carbohydrates (Baeuerle & Huttner, 1986) further indicates that sulfate moieties are critical for binding. The degree of sulfation differs between glycosaminoglycans, with heparan sulfate being the most extensively sulfated class (Esko, Kimata, & Lindahl, 2009). The interaction of FliD with the host cell receptors was observed for several *C. jejuni* isolates, consistent with the conserved nature of the protein. Bacterial attachment to heparin sulfate proteoglycans has been reported for a large array of pathogens (Bartlett & Park, 2010; Rostand & Esko, 1997), in particular *Borrelia burgdorferi* (Isaacs, 1994) and the pathogenic *Neisseria*

species (van Putten & Paul, 1995). The binding of neisserial Opa/Opc adhesins to heparin sulfate proteoglycans is followed by internalization of the bacteria (De Vries, Cole, Dankert, Frosch, & Putten, 1998). For *C. jejuni*, FliD-dependent bacterial invasion was not observed (data not shown). This may be explained by the large density of neisserial surface adhesins compared to the presence of a single FliD complex at the flagella tip. A large density of adhesins may more easily induce the clustering of host receptors that is often needed to trigger the receptor-mediated internalization (Tran Van Nhieu & Isberg, 1993).

In the flagellum of *S.* Typhimurium, the flagellar tip protein complex consists of pentamers of FliD that are assumed to form a surface-exposed plate structure with five leg domains that are buried within the flagellar structure (Vonderviszt et al., 1998; Yonekura et al., 2000). In search for the host cell binding region in *C. jejuni* FliD, we demonstrated that the N- and C-terminal domains of the protein (which may form a leg domain in *S.* Typhimurium FliD) do not carry the host cell binding domain. Instead, our results locate the binding site in the central region of the protein between the amino acids 209–418. Heparan sulfate has a high negative charge and interacts with diverse proteins including extracellular matrix proteins, growth factors, cytokines, and coagulation factors. The ability of proteins to bind heparin is largely determined by the spatial orientation of basic amino acid residues (Capila & Linhardt, 2002). The *C. jejuni* FliD protein including the region between amino acids 209–418, contains a large number of basic amino acid residues. Elucidation of the three-dimensional structure of the *C. jejuni* FliD complex may enable a more precise mapping of the amino acid residues in FliD that are critical for the contact with the cell surface heparin sulfate bearing receptors.

The function of FliD as early cell attachment factor resembles in some aspects the pilus-mediated adhesin mechanism that is present in many other pathogens. These hair-like appendages extend from the bacterial cell surface to confer initial anchoring of the bacteria to the host cells. Furthermore, the *E. coli* fimbrial tip protein FimH is conserved throughout the family *Enterobacteriaceae* and binds to carbohydrates (mannose residues) on the cell surface of host cells to promote bacterial attachment (Abraham, Sun, Dale, & Beachey, 1988) similarly to *C. jejuni* FliD. *C. jejuni* does not express pili but instead appears to exploit the flagellar tip as cellular adhesin to bind to host cell glycosaminoglycans. A major difference between the two mechanisms however is that *C. jejuni* has only two single polar flagella, which contrast with the usually large number of pili, that is expressed by a single bacterium.

Campylobacter jejuni flagella have also been implicated to act as a secretion apparatus that delivers bacterial effector molecules into the environment including the cellular cytosol (Neal-McKinney & Konkel, 2012). This mechanism resembles to some extent the classical T3SS in other bacterial pathogens such as *Yersinia*, *Shigella*, and *Salmonella*. This system encodes a needle-like structure that injects proteins into the cytosol to modulate host cell functions (Cornelis, 2006; Galán & Wolf-Watz, 2006). *C. jejuni* does not have a classical type III secretion but appears to exploit the flagella secretion apparatus to achieve the same goal (Barrero-Tobon & Hendrixson, 2014; Neal-McKinney, Christensen, & Konkel, 2010; Neal-McKinney & Konkel, 2012; Samuelson et al., 2013). The mechanism that underlies the translocation of the *C. jejuni* proteins into the cells is still elusive, but the secretion event itself seems to require neither flagellin subunits nor FliD (Neal-McKinney & Konkel, 2012). Interestingly, in *Yersinae*, the deployment of the Yop T3SS machinery appears to be triggered via binding of the bacterial LrcG protein to heparan sulfate glycosaminoglycans at the cell surface (Boyd, Sory, Iriarte, & Cornelis, 1998). It is tempting to speculate that binding of *C. jejuni* FliD to the same type of glycosaminoglycans has a similar effect on the flagella secretion machinery, but this awaits future study.

Finally, the finding that both preincubation of host cells with FliD and of *C. jejuni* with the soluble receptor analogue heparin inhibit the initial bacterial attachment strongly suggests that native flagella-associated FliD is able to interact with the identified receptor molecules on the host cells. This finding together with the high sequence conservation of FliD among *C. jejuni* strains may indicate the flagellar tip protein as a putative target for infection intervention.

4 | EXPERIMENTAL PROCEDURES

4.1 | Cell culture

Human Chang epithelial cells (ATCC CCL20.2) were routinely propagated in 25-cm^2 flask in RPMI 1640 tissue culture medium supplemented with 5% FCS at 37 °C and 5% CO_2. The human colon cell lines HT-29 (ATCC HTB-38) and HRT-18 (ATCC CCL-244) were cultured in DMEM with 10% FBS. CHO-K1 and CHO-745 cells (Esko & Stanley, 2009) were grown in RPMI + 10% FCS. CHO-lec2 and CHO-lec8 cells (Patnaik & Stanley, 2006) were kept in RPMI + Ham's-F12 (1:1) supplemented with 5% FCS.

4.2 | Bacterial culture and construction of mutants

Campylobacter jejuni strains 11168H, 81116, 108, and 81–176 were routinely grown as previously described (Bleumink-Pluym et al., 2013). In order to generate the strain 81116Δ*motAB*Δ*fliD*, we amplified the *fliD* gene and its flanking regions by polymerase chain reaction (PCR) using primers fliD-1F (5′-AGCAGGACTTGATGCTTGTG-3′) and fliD-1R (5′-GCAGCAAGAAGGGATGGTTA-3′) using genomic DNA from strain 480 as a template. The PCR product was ligated into the pGEM-T Easy vector (Promega) and introduced into *E. coli* DH5α. Reverse PCR on this vector, using the outward-oriented primers fliD-2F (5′-GTGGATCCGCAAGAGATGACTGGAAAAG-3′) and fliD-2R (5′-GCGGATCCTGAGTTAAAACCCCAGAACC-3′), was used to delete 1,650 BP from *fliD* and to introduce a unique BamHI restriction site that served to insert the Cm^r cassette from pAV35 (van Vliet et al., 1998), yielding pGEM*fliD*:cm. The plasmid was verified by sequencing. This knockout construct was then transformed into *C. jejuni* 81116Δ*motAB* as previously described (van Alphen et al., 2012) to generate 81116Δ*motAB*Δ*fliD*. The correct disruption of *FliD* was verified by PCR. The 81116Δ*flgKM* strain was constructed as described (Bleumink-Pluym et al., 1999).

4.3 | Motility assay

Motility was assessed as previously described (Radomska, Ordoñez, Wösten, Wagenaar, & Putten, 2016). Shortly, *C. jejuni* strains 81116 wild type and its mutant strains Δ*motAB*, Δ*flaAB*, Δ*fliD*, Δ*motAB*Δ*flaAB*,

and Δ*motAB*Δ*fliD* were grown overnight in HI broth. One microliter overnight culture equal to OD = 1.5 was pipetted into semisolid medium (HI medium with 0.4% agar) and bacterial migration was measured after incubation (24 hr) under microaerophilic conditions at 37 °C.

4.4 | Infection assays

Infection experiments were performed as previously described (van Putten & Paul, 1995). Cells were seeded onto 12- or 25-mm circular glass coverslips (Menzel) in a 24- or 6-well tissue culture plate, respectively, and grown to 70% confluence in 36 hr. Prior to the addition of bacteria, cells were rinsed twice with Dulbecco's PBS (DPBS) and left in Hepes buffer (20 mM Hepes, 145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and pH 7.4) supplemented with 30 mM L-serine and 5 mM glucose. *C. jejuni* (20 hr of growth at 37 °C under microaerophilic conditions) was added to the cells at a bacterium/host cell ratio (multiplicity of infection) of 100:1 and incubated for 2 hr at 37 °C at 5% CO_2. Infection was stopped by carefully rinsing the cells three times with DPBS. Live imaging was performed using a Leica SP-II confocal microscope (video). The number of attached bacteria was determined as described (van Putten & Paul, 1995). Bacterial adherence was scored blindly by examining at least 300 cells on 10 fields using a light microscope. For viability counting of cell-associated bacteria, cells were lysed cells with 1% saponin in Hepes buffer prior to plating serial dilutions on blood agar plates by 10-µl running droplets. The number of colony-forming units was determined after 2 days of growth under microaerophilic conditions.

For infection assays in the presence of recombinant FliD, the cultured cells were incubated with 25 µg of recombinant FliD for 1 hr at 20 °C and washed three times with Hepes buffer before addition of *C. jejuni*. When heparin was added to bacteria prior infection, the respective multiplicity of infection was incubated for 30 min at 20 °C with heparin before addition to the cells.

4.5 | Purification of recombinant FliD and generation of anti-FliD antibodies

The *fliD* gene (Cj0548) from *C. jejuni* 11168 was amplified with PacI and NotI overhangs (underlined) using primers 5′-CCTTAATTAAATG GCATTTGGTAGTCTATCTAGTTTAG-3′ (fwd) and 5′-CCGCGGCCGC ATTATTAGAATTGTTTGCCGCATTAATCATATTAG-3′ (rev) and directly ligated into the PCP1 vector (in house derivative of pET101) in frame with a C-terminal TEV cleavage site and a 6xHis-tag. The plasmid (named PCP1*fliD-TEV-His*) was transformed to *E. coli* BL21+. Gene expression was induced by addition of 1 mM isopropyl-beta-D-thiogalactopyranoside to 1.5 hr log phase grown cultures. After 4 hr of induction (37 °C) FliD protein was isolated under native conditions. Bacteria (of 50-ml culture) were collected by centrifugation and resuspended in 6 ml of buffer (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM imidazole, and pH 7.4) and incubated with lysozyme (1 mg/ml) for 30 min on ice. Lysed bacteria were spun (14,000 *x g*, 20 min, and 4 °C) and the supernatants were incubated (4 °C) with 4 ml of nickel-coated beads (nickel–nitrilotriacetic acid [Ni–NTA] Agarose, Thermo Scientific) for 1 hour with end-of-end rotation. NTA beads were washed with 10 ml of washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, and pH 7.4) followed by 40 ml of washing buffer +0.1% Triton-X114, and another 40 ml of washing buffer. Bound protein was eluted from the beads with 4 ml of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole, and pH 7.4) and cleaved with 2,000 U of TEV protease (T4455, Sigma; 16 hr, 4 °C, end-over-end rotation). The protein/TEV mixture was dialysed overnight at 4 °C against DPBS. The His-tagged protease was removed using Ni-NTA agarose beads. Protein concentration was measured and samples (0.3–1 mg/ml) were stored in DPBS at −20 °C.

The polyclonal antiserum reactive against *C. jejuni* FliD was generated by Eurogentec. Rabbits were immunized four times with 100 µg/ml of recombinant FliD from *C. jejuni* 11168 (without adjuvant). Titration curves were made to determine the optimal dilutions to be used in ELISA, Western blot, and immunomicroscopy.

4.6 | SDS-PAGE and Western blotting

Quality assessment of purified FliD was done by 10% SDS-PAGE followed by PageBlue staining as previously described (van Alphen et al., 2012). To determine if rabbit anti-FliD antibodies recognized FliD derived from *C. jejuni*, 10 µl of an overnight culture (OD = 1.5) from different strains was separated by SDS-PAGE and proteins transferred onto polyvinylidene difluoride membranes (PVDF; Bio-Rad). FliD protein was detected using the anti-FliD antiserum (dilution 1:1,000) in Tris-buffered saline (TBS) with 0.05% Tween (TBS-T) + 2% milk followed by secondary goat anti-rabbit immunoglobulin G (IgG) conjugated to HRP (dilution 1:10,000, Sigma). The blots were developed with ECL SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Scientific).

To quantify bacteria-associated flagellin levels, bacteria were washed with phosphate-buffered saline prior to separation on SDS-PAGE as described above. Flagellin was detected using in-house rabbit anti-FlaA antiserum (dilution 1:10,000) followed by goat anti-rabbit-HRP.

4.7 | Cell-based ELISA

Cells were grown to confluence in a 96-well plate. All incubations steps (20 °C, 1 hr, with shaking) were performed in the presence of 2% bovine serum albumin (BSA, A7030, Sigma) in DPBS and followed by three washing steps with DPBS. When appropriate, cells were grown in a 96-well plate and incubated overnight with the indicated concentrations of sodium chlorate as previously described (van Putten & Paul, 1995). Cells were fixed for 1 hr with 1% paraformaldehyde in DPBS, blocked with 4% BSA, and incubated with 20 µg/ml FliD in DPBS (unless indicated otherwise). Cell-bound FliD was detected with anti-FliD antiserum (dilution 1:10,000) and secondary goat anti-rabbit IgG-HRP (dilution 1:10,000; A4914, Sigma) using HRP-substrate (555214, BD). Absorbance was measured in a plate reader at 450 nm (Fluostar Omega, BMG Labtech).

For competition assays with soluble receptor, 20 µg/ml of FliD was preincubated with different concentrations of glycosaminoglycans using the following reagents: hyaluronic acid sodium salt (53747, Sigma), chondroitin sulfate A (C9819, Sigma), dermatan sulfate (also called chondroitin sulfate B, C3788, Sigma), or heparin sodium salt

(H4784, Sigma). Cleavage of cell surface glycosaminoglycan chains was established by treatment of the cells (1 hr, 37 °C) directly prior to the fixation step with the indicated concentrations of heparinase-I (H2519, Sigma) and chondroitinase ABC (C2905, Sigma) according to the manufactures recommendations.

To detect the binding of the biotinylated FliD proteins, we used HRP-labeled streptavidin (016-030-084, Jackson ImmunoResearch) diluted 1:50,000.

4.8 | Immunofluorescence microscopy

Cells were seeded onto 12-mm circular glass coverslips in a 24-well tissue culture plate and grown to 70% confluence in 36 hr. Cells were rinsed, incubated (1 hr, 20 °C) with 4% BSA in DPBS and then with 20 μg/ml FliD (1 hr, 20 °C) in DPBS. To visualize FliD binding to the cell surface, we subsequently stained the cells with WGA-Alexa Fluor 488 (Invitrogen) and the FliD protein with the anti-FliD antiserum (dilution 1:1,000), and goat anti-rabbit-IgG conjugated to Alexa Fluor568 (A11036, Invitrogen). Samples were viewed in an Olympus fluorescence microscope.

For visualization of bacterial adherence via the flagellar tip, cells were infected as described above with mCherry-positive *C. jejuni*. Hereto plasmid pMA5-metK-mCherry (Bouwman, Niewold, & Putten, 2013) was introduced into the relevant strains. After 1 hr of incubation, the infected cells were fixed and blocked as mentioned above. Subsequently, cells were stained with WGA-Alexa Fluor 633 (Invitrogen) and flagella with polyclonal anti-*C. jejuni* antiserum (R624, 1:1000) in combination with goat anti-rabbit-IgG conjugated to Alexa Fluor 488 (A11034, Invitrogen). Pictures were taken at 100× magnification with 2× optical zoom using the Leica SP-II confocal microscope. Brightness and contrast were adjusted using ImageJ software.

4.9 | ELISA

To show direct interaction between FliD and different classes of glycosaminoglycans, FliD protein in bicarbonate buffer (C3041, Sigma) was coated (2 μg of protein/well, 16 hr, 4 °C) onto a 96-well ELISA plates (E Bioscience, 44-2404-21). Wells were washed three times with DPBS and blocked (1 hr) with 4% BSA in DPBS. Biotinylated heparin (B9806, Sigma) was added at the indicated concentrations. After 1 hr of incubation (20 °C), HRP-streptavidin (016-030-084, Jackson ImmunoResearch) diluted (1:10,000) in 2% BSA in DPBS was added. After an additional hour of incubation, wells were washed and incubated with HRP-substrate (555214, BD) for 15 min. The reaction was stopped with 1 M H_2SO_4. Absorbance was measured in a plate reader at 450 nm (Fluorstar Omega, BMG Labtech). For the competition experiment, 50 μg/ml of glycosaminoglycans were preincubated with 5 μg/ml of biotinylated heparin (B9806, Sigma) prior to the addition to the FliD-coated wells.

4.10 | Construction and sortagging of FliD fragments

The plasmid PCP1*fliD-TEV-His* was cleaved with NotI and AscI (Thermo Scientific) to remove the TEV cleavage site. DNA sequences encoding the sortase recognition motif LPETG with a GGGS linker and cleaved NotI and AscI overhangs (underlined) with a phosphorylated 5′-end (5′-P-GGCCGCCGGTGGTGGTGGTTCTCTGCCGGAAACCGGTGG-3′ and 3′-CGGCCACCACCACCAAGAGACGGCCTTTGGCCACCGCGC-P-5′) were hybridized by heating at a one-to-one ratio (100 μM = 100 pmol/μl) for 5 min at 95 °C and then gradually (1 °C/min) cooled to 20 °C in a PCR thermocycler (Bio-Rad). The generated double-stranded DNA was ligated into PCP1, yielding PCP1*fliD-sort-His*. In order to generate FliD fragments, we digested the vector PCP1*fliD-sort-His* with NotI and PacI in order to remove the *fliD* gene. Fragments were constructed by amplifying parts of the *fliD* gene by PCR by using following primer sets with NotI and PacI restriction sites (underlined): FliDΔNC: FliDΔNC-fwd 5′-CCCTTAATTAAATGACGGTTTTTGCGAAAAG-3′ and FliDΔNC-rev 5′-AAAGCGGCCGCTTCTATAGGCTTAGAGGTTATATTTAC-3′; Fragment A: FliD-A-fwd 5′-CCCTTAATTAAATGACGGTTTTTGCGAAAAG-3′ and FliD-A-rev 5′-AAAGCGGCCGCATTCGAATCTTTTTTTCCCG-3′; Fragment B: FliD-B-fwd 5′-CCCTTAATTAAATGGGTAAATATCAAAAAGATATAAATG-3′ and FliD-B-rev 5′-AAAGCGGCCGCAATATTTGAAAAAAAACTTTCAG-3′; Fragment C: FliD-C-fwd 5′-CCCTTAATTAAATGACTAAATACGAAGACATTAATCAC-3′; and FliD-C-rev 5′-AAAGCGGCCGCTTCTATAGGCTTAGAGGTTATATTTAC-3′. The PCR products were then digested with PacI and NotI and ligated into the open PCP1*sort-His* vector. After gene expression and protein purification as described above, the products were dialyzed against TBS and biotinylated using the sortase reaction (Popp et al., 2007). FliD proteins (6.5 nmol) were incubated (16 hr, 20 °C) with equal amounts of *Staphylococcus aureus* sortase enzyme (pet30b-7 M SrtA was a gift from Hidde Ploegh, Addgene plasmid #51141) and 50× molar excess of GGGK-biotin (synthesized as described in Guimaraes et al., 2013) to site specifically biotinylate the C-terminus. Free biotin was removed by dialysis against phosphate-buffered saline (16 hr, 4 °C). The His-tagged sortase was removed using Ni-NTA beads. Biotinylation was confirmed by Western blot using Streptavidin-HRP.

4.11 | Statistical analysis

Experiments were performed at least three times in duplicate. Results were analyzed for statistical differences using the Prism GraphPad Software (version 6.05) by Student's unpaired *t* test, one- or two-way analysis of variance where appropriate. Significance was accepted at $p < .05$.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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