Functional Organization of the Autotransporter Adhesion Involved in Diffuse Adherence

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The Escherichia coli adhesin involved in diffuse adherence (AIDA-I) is a multifunctional autotransporter protein that mediates bacterial aggregation and biofilm formation, as well as adhesion and invasion of cultured epithelial cells. To elucidate the structure-function relationships of AIDA-I, we performed transposon-based linker scanning mutagenesis and constructed mutants with site-directed deletions. Twenty-nine different mutants with insertions that did not affect protein expression were obtained. Eleven mutants were deficient for one or two but not all of the functions associated with the expression of AIDA-I. Functional characterization of the transposon mutants and of an additional deletion mutant suggested that the N-terminal third of mature AIDA-I is involved in binding of this protein to cultured epithelial cells. The purified product of the putative domain could bind to cultured epithelial cells, confirming the importance of this region in adhesion. We also identified several different mutants in which invasion and adhesion were changed to different extents and two mutants in which autoaggregation and biofilm formation were also affected differently. These results suggest that although conceptually linked, adhesion and invasion, as well as autoaggregation and biofilm formation, are phenomena that may rely on distinct mechanisms when they are mediated by AIDA-I. This study sheds new light on the workings of a protein belonging to an emerging family of strikingly versatile virulence factors.

Diarrhea-causing strains of Escherichia coli are responsible for many cases of gastrointestinal disease (14). Diffusely adhering E. coli (DAEC) is one of the six classes of E. coli that have been associated with diarrhea. This group is characterized by a pattern of diffuse adherence on the surface of epithelial cells (23, 29). DAEC strains have been identified based on their diffuse adherence on cultured epithelial cells, and they appear to form a heterogeneous group. The first class of DAEC strains includes E. coli strains that harbor Afa/Dr adhesins, which are proteins that have been found to be associated with urinary tract infections and with enteric infections in infants (30). The second class of DAEC strains includes E. coli strains that express an adhesin involved in diffuse adherence (AIDA-I) (2). AIDA-I was originally identified in strain 2787 isolated from a patient with infantile diarrhea. AIDA-I has also been found in E. coli strains causing edema disease and postweaning diarrhea in piglets, two major causes of economic losses on farms worldwide (21, 24, 25).

AIDA-I belongs to the family of monomeric autotransporter proteins, a branch of the type V secretion pathway (11). Most autotransporter proteins identified so far are proven or predicted virulence factors (10). AIDA-I is synthesized as a pre-protein with a molecular mass of approximately 145 kDa and has a modular organization, like all autotransporters (1). The N terminus of the pre-protein corresponds to a sec-dependent sequence signal consisting of 49 amino acids. Cleavage of the sequence signal results in release of the proprotein in the periplasm. The proprotein comprises two different domains (34): the 100-kDa surface-exposed mature AIDA-I protein and a 45-kDa membrane-embedded domain, AIDAc. The latter is believed to form a pore in the outer membrane which may serve as the translocation conduit for mature AIDA-I towards the cell surface. Mature AIDA-I is cleaved after secretion, presumably by an autocatalytic event (34), but it remains strongly associated with AIDAc (1, 4).

Recently, AIDA-I has been proposed to be a member of a new group of autotransporter proteins called self-associating autotransporters (SAATs) (16). This group also includes Ag43, a surface protein widely distributed among pathogenic and nonpathogenic strains of E. coli and responsible for bacterial aggregation (27), and TibA, an adhesin/invasin of enterotoxigenic E. coli (5). These proteins share sequence similarities, are all glycosylated by specific heptosyltransferases that can be functionally exchanged, and possess the same multiple properties likely to be important for virulence of pathogenic strains: the ability to mediate bacterial aggregation, biofilm formation, and invasion of and adhesion to cultured epithelial cells (3, 9, 20, 22, 31–33). A structure-function study of Ag43 has been performed (15), and it revealed that the domain responsible for the aggregation phenotype resides within the N-terminal 160 residues and is distinct from the domain allowing biofilm formation. Indeed, nonaggregative variants of Ag43 could still mediate attachment to abiotic surfaces. Ag43 can also mediate adhesion to and invasion of epithelial cells (7, 31), but no region related to these phenotypes has been identified so far. In addition, this kind of analysis has never been conducted with AIDA-I or TibA. Therefore, more information is required to better understand the molecular basis of SAAT multifunctionality.
In the present report, we describe a mutagenesis study of AIDA-I. We used random transposon scanning mutagenesis and construction of a domain deletion mutant in order to identify regions in mature AIDA-I involved in the various phenotypes associated with the expression of this protein. Our data revealed that the N-terminal third of the protein is specifically involved in attachment to cultured epithelial cells. We also identified other mutations that affect each of the other functions of AIDA-I. The existence of mutations that altered invasion but not adhesion and vice versa, as well as a mutant with a mutation that altered autoggregation but not biofilm formation and vice versa, suggested that these phenomena involve distinct mechanisms. Taken together, our results provide new insights into the mechanism of action of the SAAT group of autotransporters.

### MATERIALS AND METHODS

#### Bacterial strains and plasmids. E. coli K-12 strains C600 (New England Biolabs) (F− thr−1 leuB6 thr−1 lacY1 supE44 tnaD1 bauC21) and BL21 (Stratagene) [F′ ompT hsdS (rB mB C2, and NC] deletions were introduced into pAngH by oligo-mutagenesis kit (Stratagene) using the primers listed in Table 1 and the corresponding complementary oligonucleotides, which generated pAngHΔN (deletion of amino acids 54 to 225), pAngHΔC (deletion of amino acids 224 to 637), pAngHΔC2 (deletion of amino acids 224 to 607), and pAngHΔNC (deletion of amino acids 54 to 607). To generate glutathione S-transferase (GST) fused to AIDA-I (the N-terminal portion of mature AIDA-I), a fragment of the aidA region corresponding to amino acids 50 to 224 was amplified by performing PCR with primers that introduced BamHI and XhoI restriction sites (Table 1). The DNA fragment was digested with BamHI and XhoI and cloned at the same sites into the pGEX-4T-1 vector (Amersham Biosciences) to create pGEX-AIDA-I.

#### Protein purification. The proteins were purified using an AKTA purifier system with a 5-ml glutathione Sepharose column or with a 1-ml His Trap HP column (Amersham Biosciences) according to the instructions of the manufacturer. The purity of the purified proteins was confirmed by SDS-PAGE and staining with Coomassie blue, as described below.

#### SDS-PAGE, immunoblotting, and detection of glycans. Protein samples were diluted in 2× SDS-PAGE loading buffer containing β-mercaptoethanol and denatured by heating at 100°C for 10 min. The samples were then separated by SDS-PAGE on 10 or 12% acrylamide gels. The gels were either stained with Coomassie blue or transferred to polyvinylidene fluoride membranes (Millipore). Immunodetection was performed with a serum raised against heat-extracted mature AIDA-I (a generous gift from M. Ngleka, University of Sas- katchewan) diluted 1:10,000 in blocking buffer (5% skim milk, 50 mM Tris-HCl, pH 7.5). 150 mM NaCl, 0.05% Triton X-100). A goat anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (Sigma) was used as a secondary antibody to the body according to the instructions of the manufacturer. Alternatively, an anti-HisG HRP-coupled antibody (Invitrogen) diluted 1:5,000 in blocking buffer was used for detection of proteins containing the HisG tag. Immune complexes were revealed using a 3,3′,5,5′-tetramethylbenzidine solution for membranes (Sigma).

#### Whole-cell lysates. Overnight cultures (10 ml) were grown, normalized, and centrifuged for 10 min at 12,000 × g in microcentrifuge tubes, and the pellets were resuspended in 100 μl of phosphate-buffered saline (PBS). All samples were processed by performing SDS-PAGE and immunoblotting as described below.

#### Functional assays. Autoaggregation, biofilm formation, adhesion, and invasion assays were performed as previously described (4). In the autoaggregation assay, cultures of E. coli C600 harboring the pTRC99A vector, pGEX-AIDA-I, or pGEX-AIDAN and pAah were grown until the OD600 was 0.4 and induced with 10 μM IPTG for 3 h. Bacteria were harvested, resuspended in 40 ml of Tris-buffered saline (50 mM Tris-HCl [pH 8], 150 mM NaCl) containing lysozyme (final concentration, 0.4 mg · ml⁻¹) and EDTA (pH 8) (final concentration, 10 mM), and lysed with a French press and an ultrasonic processor. The soluble fraction was isolated by 30 min of centrifugation at 16,000 × g.

For AIDA-I purification, 1-liter cultures of C600 harboring plasmids pAngH and pAah were grown until the OD600 was 0.8 and then were induced overnight with 10 μM IPTG. The cells were lysed as described above, and each lysate was centrifuged for 1 h in an ultracentrifuge at 250,000 × g. The membranes were resuspended in Tris-buffered saline containing 1% Triton X-100, incubated for 1 h, and centrifuged again. The solubilized membranes contained histidine-tagged native AIDA-I.

The proteins were purified using an AKTA purifier system with a 5-ml glutathione Sepharose column or with a 1-ml His Trap HP column (Amersham Biosciences) according to the instructions of the manufacturer. The purity of the purified proteins was confirmed by SDS-PAGE and staining with Coomassie blue.
for an additional 2 h before plating. For 1 × 10^6 CFU (total number) of bacteria expressing wild-type AIDA-I, the maximum level of adhesion was 7.9 × 10^6 CFU and the maximum level of invasion was 2.3 × 10^5 CFU.

All functional assays were performed at least three times in duplicate or triplicate. For each assay, the means of the results obtained with each mutant were compared to the mean obtained with the wild-type control by performing an analysis of variance and Dunnet posttests using the Prism 4.0 software (Graphpad Software).

Cell-based ELISA. HEP-2 cells were grown to confluence in a 96-well plate and fixed for 15 min with PBS containing 2.5% paraformaldehyde and 0.2% glutaraldehyde. After one wash with PBS, the plate was blocked with 3% bovine serum albumin in PBS for 1 h at 37°C. Native glycosylated AIDA-I, GST, unglycosylated GST-AIDAN, and glycosylated GST-AIDAN fusion proteins were added to the cells at concentrations ranging from 300 nM to 1 μM and incubated overnight at 4°C. After extensive washes with PBS, bound proteins were detected with an HRP-coupled antibody raised against GST or against native AIDA-I diluted 1:10,000 in PBS (Amersham Biosciences). Immune complexes were revealed using a 3,3',5,5'-tetramethylbenzidine solution in an enzyme-linked immunosorbent assay (ELISA) (Sigma). The background value was subtracted, and the absorption intensities were normalized by dividing the absorbance of each well by the maximal intensity measured on the plate. Experiments were conducted in duplicate at least twice. Binding curves and dissociation constants were obtained by nonlinear regression fitting to a one-binding-site model.

Immunofluorescence microscopy. HEP-2 cells were grown to confluence in a Lab-Tek II chamber slide (Nalge Nune International). After one wash with PBS, proteins (GST, unglycosylated GST-AIDAN, or glycosylated GST-AIDAN) were added to the cells at a concentration of 1 μM and incubated at 37°C for 1 h. After four washes with PBS, the cells were fixed for 15 min with PBS containing 2.5% paraformaldehyde and 0.2% glutaraldehyde, and the plate was blocked with 3% bovine serum albumin in PBS for 1 h at 37°C. Bound proteins were detected with an antibody raised against GST diluted 1:10,000 in PBS-2% bovine serum albumin. Native glycosylated AIDA-I, GST, and, as a consequence, for the cell-binding properties of AIDA-I (3). Plasmids pAngH and pAah carry compatible origins of replication and were introduced into C600.

Generation of five-amino-acid insertions in AIDA-I. For analysis of mature AIDA-I, we used a Tn7-derived transposon system that resulted in five-amino acid insertions in the protein. The mutagenesis was performed with plasmid pAngH, which allows expression of AIDA-I under the control of an IPTG-inducible promoter. In addition, AIDA-I expressed by this construct bears a HisG tag located at the N-terminal end of the proprotein. The tag did not affect the expression of AIDA-I, and bacteria expressing the tagged protein had adhesion, invasion, biofilm formation, and autoaggregation activities identical to those of bacteria expressing the wild-type untagged protein (data not shown). Glycosylation of the protein is important to ensure the normal conformation of the protein and, as a consequence, for the cell-binding properties of AIDA-I (3, 4a). We therefore used plasmid pAah, which allowed expression of Aah, the glycosyltransferase that modifies AIDA-I (3). Plasmids pAngH and pAah carry compatible origins of replication and were introduced into E. coli strain C600.

The mutagenesis procedure yielded 60 different insertions distributed throughout mature AIDA-I. Twenty-nine of these insertions did not affect protein expression, as assessed by immunoblotting of whole-cell lysates with an antibody against the HisG tag (data not shown). The insertion sites and the inserted sequences in these mutants are shown in Table S1 in the supplemental material. The remaining 31 insertions, which disrupted protein expression, were discarded. One reason for discarding these insertions is that the mutagenesis procedure left a Pmel restriction site whose translation resulted in a TAA stop codon in two of the six possible frames. Many of the insertions that resulted in a lack of protein expression were probably due to this introduction of a stop codon and therefore would not have been informative. Alternatively, some of the insertion mutations could have resulted in an unstable protein.

As described below, the function of 11 of the 29 insertion mutants was affected. We examined in greater detail the expression of these mutants, as well as a mutant that was not affected by the insertion (I8) as a control. As shown in Fig. 1, the 100-kDa mature AIDA-I was observed in whole-cell lysates of all mutant bacteria, and it was correctly located at the cell surface, as shown by the release of mature AIDA-I in the supernatant after brief heating at 60°C (1). Moreover, mature AIDA-I of all the mutants appeared at a molecular weight that represented the glycosylated form of the protein, and it could be detected with the anti-AIDA-I antiserum, which recognizes only the glycosylated protein (3). Based on these observations, we concluded that all of the insertion mutants expressed glycosylated AIDA-I, although it was still possible that the extent of glycosylation might have been slightly different in different mutants. This is in agreement with our recent finding that glycosylation is highly heterogeneous and occurs at many sites throughout AIDA-I (4a). These results therefore suggest that the insertions did not affect biogenesis, including the glycosylation status, or the structure of AIDA-I; in other words, these results suggest that these sites are structurally permissive for insertions. For the I2 and I20 mutants, however, two distinct additional bands in the whole-cell lysates reacted with the antibody against mature AIDA-I or against the HisG tag (Fig. 1A and B). These two polypeptides could also be released in the culture supernatant after brief heat treatment (Fig. 1C). N-terminal sequencing was used to identify the cleavage sites. Cleavage of both mutant proteins occurred at the transposon insertion site, between a lysine and a histidine (see Table S1 in the supplemental material). Since the additional cleavage sites were introduced by the inserted sequences and the proteins were only partially processed, we concluded that the structural integrity of these proteins was most likely not affected dramatically.

Effect of the insertions on AIDA-I. To determine the effects of the insertions on the functionality of the protein, the activity of each mutant was tested using four distinct functional assays: autoaggregation, biofilm formation, adhesion, and invasion assays. Figure 2 shows the results for all the mutants with a deficiency in one or more functions. The mutant I8 protein, which was as functional as the wild-type protein, was included as a control, and the results obtained for this mutant are shown in Fig. 2. The other mutants, for which results are not shown, were as functional in all assays as the wild-type protein.

Eleven of the 29 insertions caused a defect in one or more functions associated with the expression of AIDA-I. The positions of the insertions, as well as the results of the functional characterization analyses, are summarized in Fig. 3. The mutations in I1, I2, I4, I5, and I6, all located in the N-terminal third of mature AIDA-I, caused a defect in adhesion. As a
consequence, most of these mutations caused a defect in the ability to invade cultured epithelial cells. The only exception was mutant I4; the results for this mutant were not statistically different from the results for the wild type (Fig. 2C). These results suggest that the N-terminal region of mature AIDA-I is involved in adhesion. Three other insertions in the C-terminal half of the protein (mutants I20, I23, and I24) also caused a diminution in the ability to mediate adhesion. It is tempting to speculate that the region encompassing the positions of the mutations in these mutants could represent a second cell-binding domain in AIDA-I; however, alternatively, the mutations could indirectly affect the same binding site affected by the mutations in the N-terminal part of the protein. We also observed that bacteria expressing three different mutants, I9, I12, and I13, could adhere to cultured cells as well as bacteria expressing the wild-type protein but were unable to invade the cells. This observation therefore suggests that adhesion and invasion involve different mechanisms. Consistent with this view, but unexpectedly, we observed that invasion mediated by the I4, I23, and I24 mutants was not significantly affected, whereas the ability of these mutants to mediate adhesion was reduced, sometimes dramatically (e.g., mutant I23 [Fig. 2C]).

Only one mutant, I24, with a mutation located in the C-terminal part of mature AIDA-I, was unable to mediate bacterial aggregation, but bacteria expressing this mutant could make biofilm as well as bacteria expressing the wild-type protein (Fig. 2A and 2B). This result suggests that biofilm formation and bacterial aggregation are due to distinct mechanisms, as previously observed with Ag43 (15). Consistent with this view, one mutant with a mutation in the N-terminal part of mature AIDA-I, I9, caused a slight diminution in the ability of bacteria to form biofilms but mediated bacterial aggregation as well as the wild-type protein (Fig. 2A and 2B).

**Deletion of a putative cell-binding domain in mature AIDA-I.** Five insertion mutants with mutations clustered in the N-terminal third of mature AIDA-I affected the adhesion mediated by the protein, suggesting that there is a cell-binding domain in this region. In order to complement these results, we constructed a mutant with the N-terminal third of mature AIDA-I between residues G54 and A225 deleted (the former residue corresponds to the fourth residue of mature AIDA-I, and the latter residue corresponds to the transposon insertion site in mutant I7 [Fig. 4A]).

The correct expression and biogenesis of the ΔN mutant were checked by probing whole-cell lysates with antibodies against the HisG tag. As shown in Fig. 4B, a specific polypeptide with a molecular mass slightly greater than 75 kDa was apparent in proteins extracted from bacteria expressing the ΔN...
mutant, which is consistent with the expected size of the mature ΔN mutant (63 kDa) with glycosylation. This hypothesis was confirmed by detection of sugar using a glycan detection kit. The reaction of the ΔN mutant with the digoxigenin-hydrazine label was identical to that of the wild-type protein (Fig. 4D). In another study, we identified 16 residues of mature AIDA-I which can be modified by heptose molecules (4a). Only four of these residues are located between G54 and A225. This finding therefore explains the fact that the ΔN mutant was still glycosylated. Additionally, we performed heat extraction of bacteria expressing the ΔN mutant to verify the correct location of the protein at the cell surface (Fig. 4C). Again, a polypeptide of the expected size was apparent, without any degradation product. These assays showed that the ΔN mutant was as well expressed, glycosylated, and stable as the wild-type protein.

To assess the effects of this deletion on the functionality of AIDA-I, the four functional assays performed above were conducted again (Fig. 5). Deletion of the N-terminal third of mature AIDA-I did not affect the ability of the protein to

FIG. 2. Effect of insertion mutants on the functionality of AIDA-I. Bacteria bearing an empty vector (bars −) or expressing wild-type (bars WT) or mutated AIDA-I proteins were tested for the presence of several phenotypes. (A) Autoaggregation assay. Cultures were left standing at 4°C, and the OD₆₀₀ at the top of the culture was measured at the beginning of the assay (light gray bars) and after 180 min (dark gray bars). (B) Biofilm formation assay. Biofilms formed in microtiter plates were stained with crystal violet, and the amount of fixed dye was measured by determining the absorption at 595 nm. (C) Adhesion and invasion assays. Bacteria that adhered to HEp-2 cells were directly plated and counted. The number of intracellular bacteria was estimated by assessing the resistance to external antibiotics. The data are the percentages of adhesion or invasion compared to bacteria expressing wild-type AIDA-I. Significant differences (P < 0.05) are indicated by asterisks for aggregation and biofilm formation (A and B) and by open circles for adhesion and filled circles for invasion (C). The difference in biofilm formation mediated by I4 was not statistically significant (NS).

FIG. 3. Schematic diagram of the insertion mutations in AIDA-I and the related phenotypes: locations and effects of the mutations obtained from linker scanning mutagenesis. The open arrows indicate the insertion mutants without a functional defect, whereas the filled arrows indicate mutants with a defect for one or more functions. The filled box represents the HisG tag at the N terminus of mature AIDA-I.

FIG. 4. Location and effects of the linker scanning mutations in AIDA-I. The bars represent the presence of the mutations obtained from linker scanning mutagenesis. The open arrows indicate the insertion mutants without a functional defect, whereas the filled arrows indicate mutants with a defect for one or more functions. The filled box represents the HisG tag at the N terminus of mature AIDA-I.
mediate bacterial aggregation or biofilm formation (Fig. 5A and B). This suggests that the regions of AIDA-I involved in biofilm formation and autoaggregation are not located in the N-terminal third of the mature protein. However, the ΔN mutant was considerably less able to mediate binding to cultured epithelial cells (Fig. 5C). This is in agreement with the results of the linker scanning mutagenesis analysis that indicated that the ability of mutants I1, I2, I4, I5, and I6 to mediate adhesion was affected. This appears to contradict the hypothesis that there is a secondary cell-binding site. It should be noted, however, that the level of invasion by bacteria expressing the ΔN mutant was marginally lower than the level of invasion by

FIG. 4. Deletion of putative cell-binding domain in mature AIDA-I. (A) Schematic diagram of the deletion construct, showing plasmids pAngH (WT) and pAngHΔN (ΔN). The filled box represents the HisG tag at the N terminus of mature AIDA-I. (B) Whole-cell lysates of bacteria harboring an empty vector (lane −), expressing wild-type AIDA-I (lane WT), or expressing the ΔN mutant (lane ΔN) were obtained and probed with anti-HisG antibodies, which allowed detection of the proprotein (circles) and mature AIDA-I (arrowheads). All plasmids were transformed in bacteria expressing the Aah glycosyltransferase. (C) Heat extracts of the same cultures were obtained, resolved by SDS-PAGE, and stained with Coomassie blue. (D) Detection of glycosylation, using the digoxigenin glycan detection kit, performed with whole-cell lysates. MW, molecular mass.

FIG. 5. Function of AIDA-I bearing the ΔN deletion. An autoaggregation assay (A), a biofilm formation assay (B), an adhesion assay (C), and an invasion assay (D) were performed with cultures of C600 bearing an empty vector (bar −), plasmids pAngH and pAah (bar WT), or plasmids pAngHΔN and pAah (bar ΔN). The assays were performed as described in the legend to Fig. 2. NS, not significant.
shows that the bacteria expressing wild-type AIDA-I (Fig. 5D). This result indicates the position of the purified protein. (B) Detection of glycosylation of the purified proteins using the digoxigenin glycan detection kit. (C) Cell-based ELISA. HEp-2 cells were grown in a 96-well plate and, after fixation and blocking, were incubated with various concentrations of whole AIDA-I (■), GST (×), unglycosylated GST-AIDAN (○), or glycosylated GST-AIDAN (○). Bound proteins were detected with an antibody against GST or AIDA-I coupled to HRP. The activity of HRP was measured using a colorimetric substrate by monitoring absorption at 450 nm. The background value was subtracted, and the absorption was normalized by dividing the absorption of each well by the maximal absorption measured on the plate in order to obtain percentages of maximal binding. MW, molecular mass.

FIG. 6. Cell-binding properties of glycosylated (Glyc.) and unglycosylated (Unglyc.) GST-AIDAN. (A) Coomassie blue-stained SDS-PAGE gel of the purification products from the soluble fraction of strain BL21 bearing plasmid pGex-4T-1, pGex-AIDAN, or pGex-AIDAN and pAah obtained by performing affinity chromatography with a glutathione Sepharose column. The arrowheads indicate the glycosylation status of this protein was confirmed with a glycan conjugate.

We constructed three additional mutants with deletions in mature AIDA-I, corresponding to deletions in the C-terminal portion of the repeats (between G224 and A637 or between G224 and A667) or in all the repeats (between Gly54 and A667). These mutants were visibly expressed less than the wild-type protein or the ΔN mutant or were not expressed at all (data not shown), suggesting that the biogenesis, the stability at the cell surface, and/or the global conformation of these mutated proteins may have been disturbed. It is therefore likely that the C-terminal repeats of mature AIDA-I play a more important role in the biogenesis or the structural integrity of the protein. Since these defects were likely to influence the functional assays of the mutants, we decided not to test their functions.

N-terminal domain of mature AIDA-I, glycosylated or not glycosylated, is sufficient to mediate binding to cultured epithelial cells. To confirm that the N-terminal third of mature AIDA-I is involved in the adhesion to epithelial cells, we fused this region, corresponding to residues A50 to G224, to GST. Using affinity chromatography, we purified the GST protein alone as a control and the unglycosylated and glycosylated forms of the GST-AIDAN fusion protein by coexpression with or without the Aah glycosyltransferase (Fig. 6A). The GST-AIDAN fusion protein encompasses four glycosylation sites that we identified. The glycosylation status of this protein was confirmed with a glycan detection kit (Fig. 6B). In order to demonstrate the binding properties of the N-terminal third of mature AIDA-I, we developed a cell-based ELISA. As shown in Fig. 6C, unglycosylated and glycosylated GST-AIDAN fusion proteins bound in a saturable manner to the HEp-2 cells with equilibrium dissociation constants of 84 and 72 nM, respectively. By comparison, we observed that whole glycosylated AIDA-I could bind to the cells with a dissociation constant of 3 nM. This value is in agreement with the dissociation constant (2 nM) obtained in another study performed with the mature protein (19). The higher dissociation constants obtained with the fusion proteins (84 and 72 nM, compared to 2 or 3 nM for whole AIDA-I) could have been due to the presence of the GST moiety blocking accessibility to cellular receptors, to the difficulty of purifying a fusion protein as stable as the native protein, or to the possible presence of two binding sites in mature AIDA-I, as suggested by the results of our mutagenesis analysis.

In order to qualitatively assess the adhesion properties of the N-terminal region of mature AIDA-I and to confirm the results of the cell-based ELISA, we used an immunofluorescence microscopy technique to visualize the binding of unglycosylated or glycosylated GST-AIDAN to live cells using an antibody raised against GST. As shown in Fig. 7, unglycosylated and glycosylated GST-AIDAN bound specifically to live cells in a similar manner, whereas GST alone gave no signal. This result highlights the importance of the N-terminal domain of AIDA-I in adhesion to epithelial cells and suggests that glycosylation is not important in adhesion.

FIG. 7. Immunofluorescence detection of purified unglycosylated (Unglyc.) and glycosylated (Glyc.) GST-AIDAN on the surface of epithelial cells. Purified GST, unglycosylated GST-AIDAN, and glycosylated GST-AIDAN fusion proteins were added to confluent HEp-2 cells at a concentration of 1 μM and incubated at 37°C for 1 h. After binding of the proteins, the cells were washed, fixed, and blocked with bovine serum albumin before detection of bound proteins with antibody raised against GST. Immunocomplexes were revealed by incubation with a donkey anti-goat immunoglobulin G-rhodamine red conjugate.
DISCUSSION

In this study, we generated 29 different mutants by linker scanning mutagenesis. The positions of the mutants were distributed along the full length of mature AIDA-I. However, as shown in Fig. 8A, 27 of the 29 insertions were located in the first 700 amino acids of the protein, a region of mature AIDA-I consisting of approximately 35 imperfect repeats of a 19-amino-acid sequence shared with the other SAATs (16). By contrast, only two insertions were found in the last 200 amino acids of mature AIDA-I, suggesting that there was a bias against insertions in this part of the protein. Since we did not include the insertions that disrupted protein expression in our study, it is tempting to speculate that the region outside the repeats may be important for the structural integrity of the protein.

The structures of extracellular domains of many autotransporters are thought to consist of a β helix. A recent study proposed structural models for various autotransporter helix coils, which consist of three β strands (13). We used the model proposed for TibA (Fig. 8B), since the consensus sequence of its repeats is identical to that of AIDA-I, in order to map the transposon insertion sites in the AIDA-I mutants on the model (Fig. 8C). Interestingly, almost none of the insertions occurring in the loops connecting the strands affected function. This might have been due to the fact that loops can easily accommodate additional amino acids, compared with strands. The previously described structures of extracellular domains of autotransporters show that the β helices are stabilized by stacked amino acid residues (6, 26). An insertion in a strand might therefore displace a part of the strand either before or after the position of the stacked residues, which would act as an “anchor.” The inserted residues would then replace part of the strand and “bulge out” of the β helix. The insertion could additionally have long-distance effects by destabilizing the whole β helix. It is likely, however, that in the latter case the...
stability of the whole protein would be severely affected. It should be noted that different sequences inserted at the same location, such as the sequences of mutants I8 and I9, resulted in different phenotypes in the assays. Similarly, the I3 mutant had an insertion between amino acids G149 and H150 and behaved like the wild-type strain, whereas the neighboring insertion mutants I2 (L148-G149) and I4 (H150-A151) were deficient for adhesion. These observations suggest that both the inserted sequence and the location of the insertion influence the functionality of the resulting mutant.

As shown in Fig. 8C, the β2 strands seemed to harbor more of the insertions that did not affect function, whereas the β1 strands seemed to be more important for the functionality of the protein, as five of the six insertions in these strands disrupted one or more functions related to the expression of AIDA-I. This observation raises the possibility that the functionality of AIDA-I might be localized predominantly on one of the faces of the β helix. In this respect, we noticed that the mutations affecting adhesion and/or invasion are clustered in β1 strands and, to a lesser extent, in β2 and β3 strands, whereas the mutations that affect biofilm formation or autoaggregation are in β2 strands. This could suggest that the interactions with eukaryotic cells involve primarily the face of the β helix formed by the β1 strands, whereas autoaggregation or biofilm formation requires the face formed by the β2 strands.

The main conclusion of our study is that a cell-binding domain is present in the N-terminal third of mature AIDA-I. First, we identified five different insertions in this N-terminal region that resulted in a deficiency in adhesion to cultured epithelial cells. Second, AIDA-I with a deletion of the amino acids between G54 and A225 was unable to mediate adhesion to HEP-2 cells. Finally, the N-terminal region (between A50 and G224) fused to GST could efficiently bind to epithelial cells, as assessed by a cell-based ELISA and by immunofluorescence microscopy. The fusion protein could bind whether it was glycosylated or not. The observation that glycosylation is not required for binding is in agreement with our recent findings that glycosylation of AIDA-I is required to ensure the normal conformation of the protein and therefore might only indirectly affect the adhesion to epithelial cells (4a). Based on the known structures of other autotransporters (6, 12, 26), the N-terminal location of the primary binding domain of AIDA-I could make the interaction with a host cell receptor easier, as this part of the protein is farther away from the cell surface. The presence of a cell-binding domain at the N-terminal end of a bacterial adhesin has been reported before, for instance in the case of the Yersinia enterocolitica trimeric autotransporter adhesin YadA (28).

We also identified in the C-terminal half of mature AIDA-I three other insertion mutations that resulted in a reduction in the adhesion phenotype. Our results therefore suggest that this protein possesses two binding domains, one in the N-terminal third and one in the C-terminal half. The presence of different binding domains in the same adhesin has been described previously, for instance in trimeric autotransporters such as Haemophilus influenzae Hia (18) and in the E. coli Dr adhesin (17). As mentioned above, we noticed that most insertions affecting adhesion were located in the predicted β1 strands and to a lesser extent in the predicted β3 strands. The two faces of the β helix formed by these strands might therefore harbor the two different binding sites. Alternatively, the insertions in the C-terminal half of the protein could indirectly affect the N-terminal cell-binding domain. For instance, the N and C termini of the repeats might be spatially close in the three-dimensional structure. More work is required to distinguish between these possibilities.

Our results further suggest that invasion and adhesion rely on different mechanisms. Indeed, we identified three insertion mutants, I9, I12, and I13, which mediated binding to epithelial cells as well as the wild-type protein but were unable to mediate invasion. Invasion mediated by AIDA-I is therefore likely not to be just the effect of passive uptake by cultured epithelial cells; rather, it seems to rely on a specific mechanism. Bacteria expressing the I4, I23, and I24 insertion mutants or the ΔN deletion mutant exhibit impaired adhesion but only slightly reduced invasion. These results suggest that the binding to a receptor involved in adhesion could be different from the interactions responsible for invasion. Similarly, it was recently observed that variants of the YadA trimeric autotransporter with reduced binding to laminin exhibited greater binding to fibronectin and resulted in an increase in integrin-mediated invasion (8).

Finally, we obtained two mutants (I9 and I24) whose abilities to mediate autoaggregation and biofilm formation were not correlated; a nonaggregative mutant could mediate biofilm formation and vice versa. Separation of these two a priori related phenotypes has also been noted for Ag43 (15). Collectively, these studies therefore support the idea that biofilm formation mediated by AIDA-I involves more than the ability of bacterial cells to interact with one another.

A more precise picture of the AIDA-I autotransporter emerged from our study. Given the similarity that AIDA-I shares with Ag43 and TibA, our results are likely to apply to these and other putative SAAT proteins and shed new light on how these versatile proteins participate in the pathogenesis of diarrheagenic E. coli.

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