The Inverse Autotransporter Intimin Exports its Passenger Domain via a Hairpin Intermediate* 

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Background: Intimin exports its C-terminal passenger domain through an N-terminal β-barrel onto the bacterial surface.

Results: Insertion of an epitope tag in between the passenger and β-barrel stalls the export of the passenger domain.

Conclusion: The intimin passenger adopts a hairpin conformation during translocation.

Significance: Our results confirm the hairpin model of inverse autotransport where the passenger is translocated N-to-C.

ABSTRACT

Autotransporter proteins comprise a large family of virulence factors which consist of a β-barrel translocation unit and an extracellular effector or passenger domain. The β-barrel anchors the protein to the outer membrane of Gram-negative bacteria and facilitates the transport of the passenger domain onto the cell surface. By inserting an epitope tag into the N-terminus of the passenger domain of the inverse autotransporter Intimin, we generated a mutant defective in autotransport. Using this stalled mutant we could show that (I) at the timepoint of stalling the β-barrel appears folded, (II) the stalled autotransporter is associated with BamA and SurA, (III) the stalled Intimin is decorated with large amounts of SurA, (IV) the stalled autotransporter is not degraded by periplasmic proteases, and that (V) inverse autotransporter passenger domains are translocated by a hairpin mechanism. Our results suggest a function for the BAM complex not only in insertion and folding of the β-barrel but also for passenger translocation.

Gram-negative bacteria evolved numerous secretion systems to transport proteins across the two membranes surrounding the bacterial cytoplasm. One of these systems is the so-called type V-secretion (1, 2), representing monomeric (type Va) (3, 4) and trimeric autotransporters (type Vc) (5), two partner secretion (type Vb) (6) and the patatin-like protein PlpD (type Vd)
(7). Recently, a new family of proteins, the type Ve secretion system was described (2, 8). Two prominent members of that family are Invasin of enteropathogenic *Yersinia* strains (9) and Intimin of enteropathogenic *E.coli* strains (10, 11). Both are adhesins mediating binding to host cells and are therefore important virulence factors. Members of this family consist of an N-terminal signal peptide (12), a short periplasmic domain (13, 14) followed by a twelve-stranded \(\beta\)-barrel pore and a C-terminal passenger domain consisting of several Ig domains. This features an inverse domain order compared to classical monomeric autotransporters, which have a C-terminal \(\beta\)-barrel domain anchoring the protein to the outer membrane (2, 13). After synthesis in the cytosol, autotransporters of the type Ve are transported into the periplasm by the Sec translocon, guided through the periplasm by chaperones like SurA and finally inserted into the outer membrane via the Bam complex (8, 15, 16).

Several models try to explain the export of the passenger domain of classical monomeric autotransporters (type Va) onto the bacterial surface: according to the threading model, the N-terminus is transported first through the \(\beta\)-barrel pore (17), whereas in the hairpin model, the C-terminal part of the passenger domain forms a hairpin structure. Then folding of the passenger on the bacterial surface drives the translocation of the rest of the protein (18, 19). However, the necessity of additional energy sources has been discussed (20, 21). Another model suggests the involvement of the BAM complex not only in the insertion of the \(\beta\)-barrel domain of autotransporters, but also in the export of the passenger domain to the cell surface. This model is supported by studies in which the passenger domain of autotransporter intermediates was crosslinked to BamA (22, 23) as well as by the finding that even some folded polypeptides might be transported to the cell surface (24). In this study, we created a stalled type Ve autotransporter intermediate of the adhesin Intimin of EPEC O127:H6 by inserting a double HA tag after amino acid position 453. This position is located in the D00 domain, which comprises the N-terminal part of the extracellular passenger domain. (Fig. 1A) (25). The D00 is a protease resistant domain with unknown function. As the D00 is neither a BIG nor a C-type lectin domain, an important role in passenger domain translocation or Intimin dimerization was assumed (13, 25). With the help of this mutant, we were able to show that the \(\beta\)-barrel domain appears to be folded and stably inserted into the bacterial outer membrane although the export of the passenger domain to the cell surface is not completed yet. In addition, we could identify transient interaction partners and by different approaches we were able to confirm that the transport of the passenger domain occurs by adopting a hairpin conformation, suggesting that hairpin formation might be a general feature of all type V secretion systems.

**EXPERIMENTAL PROCEDURES**

*Bacterial strains and growth conditions-E. coli* BL21omp2 was transformed with pASK-IBA2 expression vectors containing wildtype or mutant *eaeA*, respectively. The strains were
grown at 27 °C in soy broth supplemented with a piece of autoclaved bovine liver and 100 µg/ml ampicillin (Applichem). Overnight cultures were diluted into fresh medium to an OD$_{600}$ of 0.1. Bacterial cultures were then subcultured for 2 h at 27 °C before anhydrotetracycline (AHTC) (IBA GmbH) was added at a final concentration of 200 ng/ml. To record growth curves, the OD 600 was determined every 30 min. If not stated otherwise, bacteria were then allowed to express wildtype or mutant EaeA for another 2 h. E2348/69ΔeaeA EPEC strain was grown on 37°C in LB medium.

**Site-directed mutagenesis** - To exchange single amino acids in the Intimin protein providing amber mutants, site-directed mutagenesis was used. A pair of complementary primers both including the appropriate nucleotide sequence to yield the desired amino acid were used for PCR. The derived PCR product was directly used for transformation into competent E. coli DH5α. That the sequence of the resulting plasmid was correct was verified by DNA sequencing.

**Cloning and generation of Intimin constructs** - Intimin wt and Int-HA453 mutant were constructed and cloned as described previously (8). Two C-terminal Strep-tags (GSG-SAWSHPQFEK-GSG-SAWSHPQFEK) were introduced by PCR and verified by DNA sequencing.

**Protease digestion and protein precipitation** - 2 x 10$^8$ Intimin-expressing bacteria were washed once, resuspended in PBS containing 50 µg/ml Proteinase K (Thermo Scientific) and incubated for 30 minutes on ice. To stop digestion, 4 mM phenylmethylsulfonylfluorid (PMSF) were added. To separate proteins which were released into the supernatant by PK digestion, bacteria were centrifuged for 5 min at 5000 x g and the cleared supernatant was transferred into a new tube. Subsequently proteins were precipitated by methanol and chloroform. Therefore the sample volume was adjusted to 400 µl and an equal volume of methanol as well as 300 µl of chloroform were added. After centrifugation at 13 000 rpm for 5 minutes, supernatant was rejected and 300 µl of methanol was appended to the remaining protein interphase. After an additional centrifugation step, the pellet was resuspended in SDS buffer. The samples were boiled for 10 minutes at 95°C and analyzed by SDS-PAGE and Western blot.

**Subcellular fractionation** - 50 ml of bacteria expressing Intimin wt or Intimin variants were collected by centrifugation (4500 x g, 5 min). After a washing step of the pellet, bacteria were resuspended in 500 µl resuspending buffer (0.2M Tris/HCl pH 8.0, 1M sucrose, 1mM EDTA, 1mg/ml lysozyme) with protease inhibitor (Roche) and incubated for 5 min at room temperature. Afterwards 3.2 ml H$_2$O was added for 5 min and the spheroblasts were centrifuged for 45 min at 200 000 x g. The supernatant containing the periplasmic fraction was transferred into a new tube. The pellet was resuspended in French Press buffer (10 mM Tris-HCL pH7.5, 5 mM EDTA, 0.2 mM DTT, 1 µl DNaseI (1 mg/ml), 1 mM MgCl$_2$) and cells were disrupted by French Press. Remaining
intact cells were separated by centrifugation. The cleared supernatant was ultracentrifuged for 1 h at 290,000 x g. The supernatant with the cytosolic proteins was transferred into a clean tube again and the pellet containing the membranes were resuspended in H2O.

**Crosslinks with DSP**-Two hours after induction of Intimin expression, 50 ml of bacterial cells were harvested, washed once with PBS and finally resuspended in 2.5 ml PBS with 0.5 mM of crosslinker DSP. The suspension was incubated for 30 min at room temperature on a rocking shaker and subsequently quenched with 40 mM Tris/HCl (pH 7.4). After centrifugation, outer membranes were prepared as described. For solubilizing the proteins, membrane pellets were resuspended in freshly prepared 20 mM Tris/HCl (pH 8), 0.3 M NaCl, 0.5% DDM containing buffer and incubated overnight at 4°C. After a 30 min centrifugation step (TLA55, 55,000 rpm), 100 µl of streptactin superfowl suspension was added to the supernatant for additional 2 hours. After three washing steps with buffer containing 100 mM Tris/HCl (pH 8), 150 mM NaCl, 1 mM EDTA, 0.1 % DDM, proteins were eluted with 2.5 mM desthiobiotin and SDS sample buffer.

**Sample preparation for Western blot analysis**-For preparation of whole cell lysates bacterial pellets were resuspended in H2O and SDS sample buffer to obtain 5 x 10^8 bacteria per ml and incubated for 10 min at 95 °C before loading on the gel.

**Western blot analysis**-Proteins resolved by SDS-PAGE were transferred onto nitrocellulose membranes. The membranes were blocked overnight with TBS/T (5 mM Tris_HCl, 138 mM NaCl, 0.1 % Tween-20, pH 8.0) - 5% milk powder (w/v) at 4 °C. Blots were probed with purified IgG fraction of polyclonal rabbit anti-EaeA (1:5000), anti-BamA-E (1:5000), anti-SurA (1:5000), anti-Skp (1:5000), anti-MBP (1:1000), anti-GyrA (1:1000), guinea-pig anti-DegP (1:1000), monoclonal mouse anti-HA tag, anti-Strep tagII or anti-His tag and a peroxidase-conjugated secondary anti-rabbit (diluted 1:10,000; Dianova, Hamburg, Germany), anti-mouse antibody (diluted 1:1000; Dako, Denmark) or anti-guinea pig (diluted 1:5000; Dianova). Anti-BamA and -EaeA sera had been pre-adsorbed against paraformaldehyde (PFA)-fixed bacteria deficient in the respective antigen before. As molecular weight marker, PageRuler unstained protein ladder (Thermo Scientific) was used.

**Preparation of outer membrane fractions**-Preparation of outer membranes was carried out using 50 ml bacterial culture. Cells were harvested and resuspended in 500 µl resuspension buffer (0.2 M Tris, 1 M Sucrose,1 mM EDTA, pH 8). After addition of 500 µg lysozyme (20 U/µg, MSB) and 3.2 ml water the samples were incubated for 20 min at room temperature. Protoplasts were lysed in 5 ml lysis buffer (2 % TritonX-100, 50 mM Tris, 10 mM MgCl2, pH 8) and released DNA was digested by addition of 50 µg DNaseI (10 mg/ml, Roche). Outer membranes were pelleted by centrifugation at 85,000 x g for 60 min at 4 °C. After 3 washing steps with water the membranes were resuspended in SDS sample buffer. Protein profile of abundant OMPs was visualized by staining SDS gel with coomassie brilliant blue.
Export mechanism of a type Ve autotransporter (Bio Rad) for 1 hour, followed by discoloration and recording using Odyssey imaging system (Li-cor).

**Urea extraction of outer membrane preparations**-Bacterial envelopes were pelleted by ultracentrifugation at 290 000 x g for 1 h. The membranes were resuspended in 1 ml urea urea solution (100 mM glycine, 6 M urea, 15 mM Tris-HCl, pH 7.4) and extracted for 1 h at 37 °C. Membranes from urea-treated samples were reisolated by centrifugation at 290 000 x g for 90 min at 25 °C and resuspended in SDS sample buffer.

**Immunofluorescence microscopy**-For immuno-fluorescence stainings 2 x 10^7 bacteria in PBS were centrifuged on polyethyleneimine-coated coverslips, fixed for 30 min with 4 % PFA in PBS (w/v) and subsequently blocked with 1 % bovine serum albumine (BSA) in PBS (w/v) at room temperature. For stainings of periplasmic localized antigens, bacterial cell walls were permeabilized for 20 min in 0.5 % Triton X-100/PBS (v/v). Stainings were performed using preabsorbed polyclonal rabbit antibodies directed against EaeA (provided by Prof. Gad Frankel, London, UK) (diluted 1:200) and a 1:200 dilution of a Cy2-conjugated secondary anti-rabbit antibody (Dianova, Hamburg, Germany). StrepTagII (diluted 1:100) or HA-tag (diluted 1:100) were stained with corresponding monoclonal mouse antibodies and Cy3-conjugated secondary anti-mouse antibody in a 1:100 dilution (Dianova, Hamburg, Germany). Secondary antibodies were incubated at room temperature for 2 h in a dark chamber. Finally, coverslips were mounted with Mowiol. Fluorescence images were obtained using an upright DMRE fluorescence microscope (Leica, Wetzlar, Germany) equipped with a Leica b/w digital camera using the 100x objective, optovar 1.6x and the software Leica application suite. All samples within one experiment were recorded at identical software settings (exposure, gamma correction). Images were processed and assembled into figures using Adobe Photoshop.

**Quantification of Intimin surface localisation by flow cytometry**-Two hours after start of Intimin protein expression, 5x10^7 bacteria were harvested by centrifugation. Cells were washed with PBS, fixed with 4% PFA and finally blocked with 1% BSA in PBS. Afterwards, cells were stained with rabbit anti-Intimin (1:200), mouse anti-HA tag or mouse anti-Strep tag antibodies overnight at 4°C followed by an incubation with anti-rabbit Cy2- (1:100; Dianova) or anti-mouse Dylight649-conjugated (1:100; Jackson, Newmarket, UK) secondary antibody for 2 h at room temperature. Surface localization of Intimin C-terminal domain, HA tag or Strep tag was measured by flow cytometry using an LSRFortessa cell analyzer (Becton Dickinson, Heidelberg, Germany). Data were analyzed with WinMDI (J. Trotter) software. The mean fluorescence intensity of three independent experiments is shown.

**Intimin adhesion assay**-1,5 x 10^5 HeLa cells (ATCC number: CCL-2) were seeded onto coverslips and grown overnight in RPMI-1640 (Biochrom, Berlin, Germany) supplemented with 10 % fetal calf serum (FCS; Gibco,
Darmstadt, Germany) and 1 % penicillin/streptomycin (Pen/Strep). Next day cells were washed twice and incubated in medium without antibiotics at 37 °C and 5 % CO₂ for one hour before preinfection with E. coli E2348/69 ΔeaeA EPEC strain (provided by Prof. Gad Frankel, London, UK). Overnight cultures of the EPEC eaeA mutant strain were subcultivated for 2 h at 37 °C, harvested by centrifugation (4000 x g, 5 min) and washed once with PBS. Then, HeLa cells were infected at a MOI of 100. Bacteria were centrifuged onto the cells at 300 x g for 2 min and incubated for 2 h at 37 °C, 5 % CO₂ followed by 4 washing steps. Remaining adherent bacteria were killed by incubation with gentamicin (100 µg/ml) for 1 h. Finally, cells were washed with medium without antibiotics. For infection with E. coli omp2 strains expressing the wildtype or mutant Intimin variants, bacteria were subcultivated as described above. After 2 hours of protein expression, bacteria were harvested and washed once with PBS. The preinfected HeLa cells were then infected at MOI 100 for 2 h. Following 3 washing steps with PBS, the cells were fixed overnight with 4 % PFA in PBS. After staining of cells with fuchsine for 30 seconds, the coverslips were finally mounted in Entellan (Merck, Darmstadt, Germany) and analyzed with a light microscope with a 100-fold magnification.

RESULTS

Insertion of a 2xHA-tag after aa453 into the Intimin passenger domain abolishes adhesion to Tir-primed Hela cells—In a previous study (8), we analyzed the topology of the Intimin membrane anchor by insertion of tandem HA epitope tags into (i) loops and turns, into (ii) the periplasmic domain at the N-terminus, (iii) the α-helical linker between β-barrel and passenger domain and, (iv) the passenger domain. By the insertion of a tandem HA epitope tag (Fig. 1A) after amino acid 453 into the passenger domain of Intimin (IntHA453) we created an Intimin variant producing an interesting adhesion phenotype (described below). Intimin as an adhesin specifically binds to the translocated intimin receptor (Tir). Tir is injected into host cells by enteropathogenic E. coli (EPEC) (10, 27) via a type III secretion system. To test the adhesive properties of IntHA453 as a functional readout for surface display of the passenger, we analysed the ability of IntHA453 to mediate adherence to the Tir receptor. Therefore, HeLa cells were primed with the Tir receptor by an Intimin deficient EPEC strain (EPEC ΔeaeA). Using the method established in Oberhettinger et al. (2012), the same cells were infected with E. coli BL21 omp2 expressing wild-type Intimin (Int wt), IntHA453, or C-terminally Strep-tagged versions (Fig. 1A) thereof. As shown in Figure 1B, bacteria expressing Int wt or Int wt-Strep were able to resist being removed by extensive washing steps, indicating adhesion to the host cells via the Tir receptor. In contrast, bacteria expressing IntHA453 were efficiently removed by washing which is indicative of weak or no binding. Moreover, our data demonstrate that a C-terminally attached Strep tag does per se not disturb the adhesive properties (and thus passenger translocation) of Intimin as we detected comparable numbers of
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bacteria expressing Int wt or Int wt-Strep binding to Tir-primed cells. As a control for efficient removal of EPEC ΔeaeA which was used for priming the cells with the Tir receptor, HeLa cells which were only preinfected are depicted (EPEC ΔeaeA). E. coli BL21omp2 carrying an empty vector (pASK-IBA2) was included as a negative control for binding to Tir-primed cells. Taken together our findings indicate that IntHA453 is not able to mediate adhesion of bacteria to host cells carrying the Tir receptor.

IntHA453 is expressed at wildtype level and the β-barrel domain appears folded and stably inserted into the outer membrane. In the previous section we have shown that IntHA453 is not able to mediate adhesion to Tir-primed HeLa cells. There are at least two possible explanations for this phenotype: as we know that autotransporter-mediated adhesion is very much a dose-dependent effect (28, 29), the protein expression level of IntHA453 might be reduced compared to Int wt. Alternatively, the IntHA453 is expressed at wild-type levels, but it is either masked or misfolded and thus not able to adhere to the Tir receptor. To test these hypotheses, we analysed the protein expression levels by western blot, the folding of the β-barrel by heat modifiability assays and a stable outer membrane insertion by urea extraction. As shown by western blots with whole cell lysates and by using antibodies directed against the C-terminal part of the passenger domain of Intimin (Figure 2A upper panel), the C-terminal Streptag (middle panel) or the HA-epitope tag (lower panel), Int wt, Int wt-Strep, Int HA453 and Int HA453-Strep are expressed in comparable amounts. All proteins had an apparent molecular weight of ~ 100 kDa which is in accordance with the calculated molecular weight. We did not observe degradation products with any of the Intimin variants. Taken together, our results demonstrate that the expression level of Int HA453 is comparable to the wild-type and thus is not the cause of the altered adhesion behaviour.

In order to examine if the Intimin protein variants are correctly folded and inserted into the lipid bilayer, we first prepared outer membrane fractions of E. coli BL21 omp2 expressing the indicated proteins. Then, we performed a heat modifiability experiment, a typical assay to assess the folding of β-barrel proteins (8, 30). As shown in Figure 2B, all Intimin variants migrate faster through the SDS gel after heating at 50°C. This species represents the folded form of the β-barrel. Upon heating to 95°C the running behaviour is altered due to denaturation of the entire protein. This result is remarkable especially for the IntHA453 mutants, because (I) the inserted tags do not interfere with β-barrel folding and (II) the folding of the β-barrel obviously is completed (this will be of interest later on).

In addition to the heat modifiability assay, we tested if the Intimin variants are correctly inserted into the lipid bilayer or only loosely attached to the membrane by weak hydrophobic interactions. Therefore, membrane protein fractions were extracted with 6 M urea and subsequently the pellet fraction (P) containing the fully integrated insoluble proteins- was
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separated from the supernatant (S) -containing the extractable proteins-. As shown in Figure 2C, the major fraction of the tested proteins was found in the pellet fraction. A minor fraction of the proteins was extractable by urea, conceivably due to the overexpression conditions we have used. Thus, we can conclude that IntHA453 is folded and inserted into the outer membrane in comparable amounts as Int wt. This indicates that the loss of adhesive properties was not due to reduced assembly efficiency and/or reduced insertion into the outer membrane.

Surface display of the passenger domain is impaired in Int HA453-In order to test if the passenger domain is localized on the cell surface we performed immunofluorescence experiments. By using an antibody directed against the most C-terminal 280 aa (26) (Fig. 1A) we are able to detect the Intimin passenger only. Moreover, by omitting a permeabilization step prior to the application of the antibody, we can detect the passenger only if it is exposed on the bacterial surface (8). We found that bacteria expressing Int wt or Int wt-Strep showed a ring-shaped outer membrane staining with the anti-Intimin antibody (Fig. 3A). This finding indicates surface exposure of the passenger domain. However, IntHA453 as well as the Strep-tagged variant thereof (IntHA453-Strep) yielded a significantly reduced fluorescence signal (Fig 3A, upper panel). To our surprise, when we performed the same assay with antibodies directed against the HA-epitope tag, we were able to detect it on the bacterial surface (Fig. 3A, middle panel). Neither an unfolded but exported passenger (which would be detected by the Int antibody on the surface) nor degradation of the passenger (which was excluded in the section above) can explain this effect. Thus, we assumed that by inserting the tandem HA-tag we have generated a stalled autotransport intermediate, in which passenger translocation was initiated but the process was interrupted before passenger export was complete. If then transport of the passenger is initiated by the formation of a hairpin, one would be able to detect the HA-tag on the bacterial surface, but not the C-terminal part of the passenger domain. This hypothesis was supported by the fact that the most C-terminal Strep tag, which is surface exposed for Int wt-Strep and which does not interfere with autotransport (Fig. 3A, lower panel), is not detectable on the cell surface in bacteria expressing Int HA453-Strep. All these findings were corroborated and quantified by fluorescence-activated cell sorting (FACS) (Fig. 3B). From these data we hypothesized that IntHA453 might be a stalled autotransport intermediate adopting a topology as illustrated in Figure 3C. This intermediate arised probably due to the disruption of the D00 domain of Intimin. Position 453 lies within the first predicted β-strand of the N-terminal extracellular domain (D00) of the passenger. Thus, the introduced double HA tag presumably leads to misfolding of the D00 domain. Tsai et al. (13) assumed that this domain could function as an autochaperone domain as it was described for classical monomeric autotransporters (31-33). Such domains initiate the vectorial export of the passenger by forming a hairpin
intermediate and improve transport efficiency. This could explain why the insertion of the double HA tag leads to stalling.

However, to confirm this hypothesis we had to test by other means if our topology model really holds true.

The C-terminal part of the passenger domain of Int HA453 can be cleaved off the N-terminal β-barrel by Proteinase K treatment, but is protected from further degradation. To analyze the putative autotransport intermediate in more detail and to characterize the topology of the C-terminal passenger domain, we performed proteolytic treatment with Proteinase K (PK). As reported elsewhere (15), Int wt as well as Int wt-Strep are resistant to protease, as PK treatment leads to a slight decrease only in the total amount of protein. Compared to Int wt, IntHA453 was highly sensitive to PK treatment (Fig. 4A). Whereas the signal for the full length protein quantitatively disappeared with Int HA453 and Int HA453-Strep, we could observe the formation of a fragment of about ~55 kDa (from now on termed PK fragment). The PK fragment was not only detectable with the Intimin antibody indicating that the fragment contains at least parts of the Intimin antibody binding site, i.e. the passenger domain (depicted in Fig. 1A), but under the chosen experimental conditions it was also protected from further degradation. Immunofluorescence staining of PK-treated bacteria expressing the Intimin variants using the anti-Intimin antibody confirmed the protease resistance of Int wt and Int wt-Strep, as the outer membrane still displayed ring-shaped staining. However, IntHA453 and Int HA453-Strep could not be detected on the surface of untreated as well as PK-treated bacteria using the anti-Intimin antibody (Fig. 4B).

As the tandem HA tag is intrinsically unfolded, we assumed that it might be the structure which is proteolysed by PK. To investigate that, we used bacteria producing IntHA453 or IntHA453-Strep and treated them with PK. Then, whole cell lysates were prepared and analysed by Western blot and immunofluorescence staining using antibodies directed against the HA-tag. We found that the HA tag was no longer detectable after treatment with PK as detected by Western blotting (Fig. 4C, left panel). Digestion of the HA tag also resulted in a loss of fluorescence signal on the cell surface of E. coli (Fig. 4C, right panel). As our Intimin antibody recognizes the last 280 amino acids of the C-terminal passenger domain, we assumed that the ~55 kDa fragment, which is detectable with the Intimin antibody, should also be detectable with the Strep tag antibody, as the tag is located at the very C-terminus of the protein. To test this assumption, we reprobed whole cell lysates of Int wt-Strep and IntHA453-Strep (used in Fig. 4A) with antibodies directed against the Strep tag. PK treatment of bacteria expressing Int wt-Strep leads to degradation of the C-terminal Strep tag (Fig. 4D, left panel) and additionally results in a loss of fluorescence signal at the bacterial surface (Fig. 4D, right panel). In contrast, the PK fragment arising after proteolysis of IntHA453-Strep was detectable with the Strep tag antibody (Fig. 4D, left panel; labeled with an
This finding confirmed our hypothesis that the PK fragment comprises the most C-terminal part of the Intimin passenger domain. In summary, insertion of the tandem HA tag at position 453 results in destabilisation of the Intimin passenger domain and renders the protein accessible to PK. However, the PK fragment which is generated is protected from further degradation and obviously inaccessible to antibody binding without permeabilization of the outer membrane.

The ~55 kDa PK fragment is localized in the periplasm. Proteinase K treatment of bacteria expressing IntHA453 leads to the formation of a ~55 kDa Intimin fragment, which can be recognized by Western blot analysis with the Intimin as well as the Strep tag antibody (Fig. 4A and 4D). However, because we analysed whole cell lysates, we were not able to discern if the fragment might be released into the supernatant or if it was still associated with the bacteria (which would be the case if the fragment resides in the periplasm). To resolve this question, we again expressed IntHA453-Strep in E.coli BL21omp2 and treated the bacteria with PK. Bacteria expressing Int wt-Strep served as control. Afterwards we separated a pellet fraction containing unsoluble material and a supernatant containing soluble proteins. Here, Int wt-Strep, which is inaccessible to PK was found exclusively in the pellet fraction (Fig. 5A). However, the PK fragment was solely found in the pellet fraction and could not be detected in the supernatant (Fig. 5A.). This indicates that the PK fragment is not quantitatively released into the supernatant, but somehow stays associated with the bacteria.

The IntHA453 passenger domain is stalled in a hairpin conformation-To test whether inverse (Ve) autotransporters adapt a similar hairpin conformation as discussed for the initiation of Va (13) and Vc (34) autotransport, we expressed Int wt, Int wt-Strep, Int HA453 and Int HA453-Strep in E.coli and performed
immunofluorescence microscopy. Native bacterial cells were either treated with TritonX-100 to permeabilize the outer membrane or we omitted the permeabilization step. After that, bacteria were incubated with antibodies directed against the C-terminally attached Strep tag. As shown in figure 6, the Strep antibody did not produce any unspecific signal with bacteria expressing Int wt or IntHA453 irrespective of the treatment with TritonX-100. However, staining of bacteria expressing Int wt-Strep resulted in a ring-shaped peripheral fluorescence even without permeabilization. Permeabilized cells showed comparable outer membrane staining. In contrast to this, using bacteria expressing Int HA453-Strep, the Strep tag was detectable only if the outer membrane was permeabilized and allowed the antibodies to enter the periplasmic space (Fig. 6). Bacteria expressing IntHA453 did not give a fluorescence signal, demonstrating that the signal we obtained with IntHA453-Strep is not due to unspecific binding of the anti-Strep antibody to other periplasmic content. In summary, our data obtained by immunofluorescence staining as well as treatment of bacteria with PK clearly demonstrate that the passenger domain translocation of the Int HA453 is stalled in a hairpin conformation at a point where the N-terminal part of the passenger (comprising the HA-tag) is already surface exposed, whereas the C-terminus is still located in the periplasm.

IntHA453 copurifies with components of the β-barrel assembly machinery and the periplasmic chaperone SurA after chemical crosslinking.

Insertion of Intimin into the outer membrane of E. coli depends on BamA and it has been shown that the loss of SurA leads to an accumulation of the membrane anchor domain of Intimin in the periplasm (15). We have reported previously, that Invasin, an inverse autotransporter adhesin of Yersinia enterocolitica, is no longer inserted into the outer membrane under either BamA-depletion or SurA-deletion conditions. In our experimental setting, the periplasmic chaperone-protease DegP procured the complete degradation of Invasin within the periplasm. As a result the protein was no longer detectable in whole cell lysates (8). In the present study, we wanted to find out if in vivo Intimin directly interacts with the periplasmic chaperone SurA and also the β-barrel assembly machinery during its biogenesis. Moreover, we were interested to find out if these interactions are somehow altered in the stalled autotransporter mutant IntHA453-Strep. To do so, we used Dithiobis[succinimidylpropionate] (DSP), a thiol-cleavable, membrane-permeant and amine-reactive crosslinker. After crosslinking and solubilization with detergent, we enriched proteins carrying the Strep-tag (and proteins crosslinked to those) by affinity purification using Streptavidin-coated beads. The resulting eluates were heated in the presence of Dithiothreitol (DTT)-containing laemmli buffer to reduce the disulfide bond in the spacer arm of DSP and thereby disrupt the crosslinks. Finally all samples were analyzed by SDS-PAGE and Western blot. As a negative control we included bacteria harbouring an empty pASK-IBA2 vector. As shown by
Western blot using the anti-Intimin antibody, both Int wt-Strep as well as IntHA453-Strep were recovered well from the solubilized membrane fractions, with the enrichment of Int wt-Strep being slightly more efficient (Fig. 7A). Treatment of the samples with DSP did not significantly affect the efficiency of enrichment of Int wt-Strep or Int HA453-Strep via the Strep-tag. Next we analysed the identical samples for the presence of BamA and periplasmic chaperones. Without DSP, neither BamA nor periplasmic chaperones copurified with Int wt-Strep, whereas addition of DSP lead to crosslinking of Int wt-Strep to BamA as well as to the chaperones Skp and SurA. Furthermore, we could detect all other BAM complex proteins (BamB, BamC, BamD and BamE) in samples where BamA was crosslinked.

Whereas the periplasmic chaperone Skp did not reveal enhanced crosslinking to the stalled autotransport intermediate IntHA453-Strep, the much more SurA was crosslinked to the stalled mutant compared to Int wt-Strep. This is in accordance to previous data which showed a specialized role for SurA in passenger domain secretion, whereas Skp has a role only at early stage of β-barrel assembly, which seems folded and assembled for Int wt as well as IntHA453 mutants. Moreover, the interaction with BamA varied significantly: for IntHA453-Strep an interaction to BamA was visible already without adding a crosslinker, although such a contact between BamA and a substrate protein like Intimin is expected to be very transient. Addition of DSP enhanced the amount of copurified BamA with IntHA453-Strep drastically and also the levels of immunoprecipitated BamB-E. E. coli cells harbouring only the empty vector pASK-IBA2 served as negative controls for the immune-precipitation and confirmed the specificity of the antibodies used. To rule out that the high protein levels of SurA and the BAM complex components crosslinked to IntHA453-Strep were only due to upregulation of the corresponding genes, we analyzed the expression in whole cell lysates. The steady state levels of the periplasmic chaperones Skp, DegP and SurA as well as the BAM complex components BamA-E are comparable independently of which Intimin construct was expressed heterologously (Fig. 8).

In summary, our crosslinking data confirm the idea of a stalled autotransport intermediate. Although interactions between outer membrane proteins like Intimin with periplasmic chaperones and the BAM complex are very short-lived, addition of DSP can trap also such transient protein-protein contacts. Because the biogenesis of the stalled autotransport intermediate is not completed yet, IntHA453-Strep is still in contact to chaperones and the BAM complex as Int wt-Strep, attempting to facilitate export of the passenger domain to the cell surface and thus to complete protein biogenesis.

DISCUSSION

*E. coli* Intimin is a prototypical inverse autotransporter, also called type Ve secretion system (2, 8). By introducing an HA tag after position 453 in Intimin, we have produced a
mutant of an inverse autotransporter that is stalled in autotransport. We were able to show that the β-barrel domain is properly inserted in the outer membrane just as in the wildtype situation, and is folded according to gel-shift assays. In contrast, the passenger domain is not located on the cell surface in the mutant, as only the introduced HA tag can be stained with antibodies in unpermeabilized cells, while the antibodies specific to the C-terminus of the passenger only yield a (periplasmic) fluorescence signal after cell permeabilization with detergent. The stalled autotransporter interacts both with BamA and with the periplasmic chaperone SurA, shown by crosslinking experiments, and in accordance with data on type Va autotransporters where SurA has a special role in passenger secretion (35). The massive decoration with SurA suggests an unfolded conformation of the passenger, but at the same time might explain why the protein is protected from proteolysis. It seems that the stalled autotransporter does not trigger the periplasmic stress response under the conditions used in this work (Fig. 8). This is corroborated by the fact that bacteria expressing the stalled autotransporter do neither have a growth defect nor the OMP profile is significantly changed compared to bacteria expressing the wildtypic Intimin (Fig. 9).

It is widely accepted that autotransport proceeds through the transmembrane β-barrel of the translocation domain via a hairpin intermediate (18, 36), and that this is true for all type V secretion systems with the possible exception of two-partner secretion (type Vb), where the passenger might also be threaded through N-terminus first (37). Thus, the only explanation for the labeling results where the HA tag is on the cell surface while the rest of the passenger remains in the periplasm is the formation of a hairpin intermediate (Fig. 3C). For classical autotransporters (type Va), it has been suggested that β-barrel insertion and initiation of hairpin formation are coupled, and that possibly the barrel is not fully formed when autotransport starts(23, 35, 38). Our data on the type Ve autotransporter Intimin shows a presumably folded barrel with a stalled hairpin – not necessarily contradicting a simultaneous insertion of barrel and hairpin into the membrane, but strongly suggesting that the rest of the autotransport process proceeds with a fully formed barrel present.

The Bam complex is the protein complex that facilitates the membrane insertion and folding of practically all transmembrane β-barrel proteins into the Gram-negative bacterial outer membrane. It is an essential outer membrane component, albeit transmembrane β-barrel proteins readily insert and fold into lipid bilayers in vitro completely autonomously (39). Thus, the essential role of the Bam complex in the cell is presumably the lowering of the activation energy for membrane insertion and thus the improvement of insertion kinetics, to avoid accumulation of unfolded proteins in the periplasm. In addition, the POTRA domains of BamA have been shown to act as chaperones, interacting with amphiphatic β-strands (40). The Bam complex is also necessary for in vivo insertion of autotransporters that have the same
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characteristics and the same C-terminal insertion signal as other transmembrane β-barrel proteins (41, 42). The direct involvement of the Bam complex in the biogenesis of autotransporters has been shown in detail for type Va (23, 35, 43) and the trimeric Vc (44) secretion systems, as well as for inverse autotransporters (Ve) (8). If and how the Bam complex is also important for hairpin formation is unclear. Recent structural and functional data on the Bam complex suggests that a hybrid barrel is formed between the BamA barrel and the substrate (45-48). From this intermediate, the substrate barrel is then released in a process that can be described as “budding”. Note that our data shows a presumably folded barrel in the membrane for the stalled Intimin autotransport mutant, strongly suggesting that this budding is completed while autotransport is not. Several scenarios are conceivable that would explain the observed interaction of the mutant with BamA (Fig. 10):

(I) The barrel is formed with the help of BamA, but autotransport (and even hairpin formation) is only initiated afterwards. This is somewhat in contrast to observations for type Va autotransporters where barrel insertion and hairpin formation are thought to be coupled (22, 23) (Fig. 10A). (II) The barrel is formed and during the hybrid barrel stage, the hairpin is inserted. This can then lead to two different outcomes: one end can stay in the BamA barrel (Fig. 10B), or both ends of the hairpin can end up in the autotransporter barrel (Fig. 10C). In all three cases, the (stalled) passenger domain could still interact with the POTRA domains of BamA, and in some of the cases possibly also with other parts of BamA (e.g. with the inside of the BamA barrel), which would explain the crosslinking data presented in this work. Much more detailed interaction studies will be necessary to elucidate the exact sequence of events in autotransport.

It is worth noticing that the trimeric autotransporters also depend on BamA for membrane insertion, and also seem to form hairpin intermediates (34, 44, 49). It is hard to conceive how three hairpins could be formed, and three parts of a transmembrane β-barrel assembled and inserted simultaneously by one Bam complex, and three individual Bam complexes are difficult to imagine to work in a synchronous fashion. The current challenge in the field of autotransport is thus to figure out how universal the hairpin mechanism is for all autotransporters, from Type Va to Type Ve.
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FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. Int HA453 is not able to mediate adhesion of E.coli to HeLa cells carrying the Tir receptor. (A) Domains of EPEC Intimin. Amino acids comprising the individual domains are depicted, insertion sites and sequence of the HA- (purple) as well as of the Strep-tag (blue) is constituted, Intimin antibody binding site is labeled in green. (B) HeLa cells were preinfected with EPECΔaeaeA that injected the Tir receptor into the host cell membrane. Subsequently, HeLa cells were infected with E.coli BL21 omp2 expressing Intimin wt (Int wt), the HA-tagged variant Int HA453, and the Strep-tagged version thereof (Int wt-Strep and Int HA453-Strep). Adhesion is only possible if the C-terminus of Intimin is correctly folded and exposed on the bacterial cell surface. Proper adhesion was only observed for bacteria expressing Int wt or Int wt-Strep. Scale bar corresponds to 2 μm.
FIGURE 2. Expression and assembly of wild-type Intimin and Strept/HAtagged variants in the bacterial outer membrane. (A) Expression of Intimin constructs in E.coli BL21omp2 was induced by adding AHTC. Protein profiles of whole cell lysates were analyzed by Western blotting and immunodetected with antibodies against Intimin, Strept- or HA-tag. (B) Heat shift. Correct folding of the β-barrels from Intimin wt and Intimin variants was analyzed by heating outer membrane fractions at 50°C or 95°C respectively. Properly folded β-barrels show heat modifiability due to denaturation only at higher temperature. (C) Urea extraction. To distinguish between fully membrane inserted and loosely attached membrane proteins, outer membrane fractions were incubated with 6M urea. Afterwards insoluble material (P = pellet) was separated by ultracentrifugation from the supernatant (S) containing the soluble protein fraction.

FIGURE 3. Insertion of a HA-tag at position 453 of E. coli O127:H6 E2348/69 Intimin results in a stalled autotransport intermediate. (A) Immunofluorescence staining of E.coli BL21 omp2 expressing Intimin wt and Intimin mutants. Bacteria were fixed and incubated with either Intimin antibody recognizing the C-terminus, anti HA-tag or anti Strep-tag antibody. Scale bar corresponds to 5 µm. (B) Flow cytometry analysis. Surface exposure of the binding sites for Intimin, HA-tag and Strep-tag antibodies was assessed and plotted in mean fluorescence intensity for the indicated constructs, which were expressed in E.coli BL21 omp2. (C) Schematic illustration of the analyzed Intimin constructs. The binding site for the Intimin antibody at the C-terminus of the protein is marked in green, the Strep-tag is coloured in blue. The HA-tag which causes the stalled phenotype is labeled in red.

FIGURE 4. PK digestion of Intimin mutants expressed in E.coli leads to the release of a protected C-terminal fragment. (A) Formation of a ~55 kDa PK fragment. Bacteria expressing Int wt, IntHA453 and Streptagged variants were treated with Proteinase K (PK) for 30 min. After addition of PMSF as protease inhibitor, whole cell lysates were subjected to SDS-PAGE and Western blot analysis with anti-Intimin antibody was performed. The generated PK fragment is depicted and only visible for IntHA453 mutants. (B) Immunofluorescence staining of PK treated bacteria. Bacteria from (A) were labeled with anti-Intimin antibody after or without treatment with PK. (C) The HA-tag is cleaved by PK. Int HA453 and IntHA453-Strep samples from (A) were analyzed by Western blot and immunofluorescence immunodetected with anti-HA tag antibody. Outer membrane staining was only observed for untreated bacteria. (D) The PK fragment can be recognized by C-terminal Streptag. Streptag of Int wt-Strep was cleaved after addition of PK, whereas treatment of IntHA453-Strep resulted in the formation of the protected ~55 kDa fragment, which can be detected with anti-Strep tag antibody in Western blot analysis. All scale bars correspond to 5 µm.

FIGURE 5. The protected PK fragment is localized in the periplasm. (A) E.coli BL21 omp2 expressing Int wt-Strep or IntHA453-Strep respectively were analyzed directly (whole) or treated with PK. Afterwards bacteria were centrifuged and the pellet as well as the supernatant (SN) fractions were analyzed by SDS-PAGE and Western blotting using anti-Intimin antibody. (B) Subcellular fractionation of E.coli BL21 omp2 expressing Int wt-Strep or IntHA453-Strep after PK treatment. Membrane (Mem), periplasmic (Peri) as well as cytosolic (Cyto) fractions were analyzed by SDS-PAGE and Western blot with antibodies directed against Intimin. BamA, an outer membrane protein, MBP, a periplasmic protein and DnaK as a cytosolic protein were used as controls showing the purity of the different fractions.

FIGURE 6. Immunofluorescence staining of C-terminally Streptagged IntHA453 AT intermediate indicates a hairpin conformation of the passenger domain. Int wt or IntHA453 as well as Streptagged variants were expressed in E.coli BL21 omp2. Bacterial outer membrane was permeabilized (upper panel) or not (lower panel) with Triton X-100 prior staining with anti-Strep tag primary antibody. Scale bar corresponds to 5 µm.

FIGURE 7. Stalled translocation of the passenger domain allows the identification of transient interaction partners. (A) Interaction with BAM complex components and (B) periplasmic chaperones. Bacteria expressing Int wt-Strep or IntHA453-Strep were either incubated with the membrane permeable
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crosslinker DSP (+) or left untreated (-). Afterwards outer membrane fractions were isolated, outer
membrane proteins were solubilized with DDM and Strep-tagged Intimin was purified with Streptavidin
beads. Proteins were eluted from the beads with desthiobiotin and analyzed by Western blot analysis.

FIGURE 8. Protein expression levels of BAM complex components and periplasmic chaperones. (A) Whole cell lysates of E.coli BL21 omp2 expressing Int wt or Intimin variants were analyzed for the expression of BAM complex components BamA-BamE. Samples were taken 2h after start of Intimin protein expression. (B) Protein levels of the periplasmic chaperones Skp, DegP and SurA in whole cell lysates.

FIGURE 9. Growth and outer membrane protein profile of bacteria overexpressing Int HA453-Strep. (A) Expression of the stalled autotransporter does not produce a growth phenotype in bacteria grown at 27°C. (B) Coomassie gel and Western blot with anti-Intimin antibodies. The OMP profile remains unchanged upon overexpression of the stalled autotransporter. Expression levels of Int wt, Int wt-Strep, Int HA453 and Int HA453-Strep are comparable.

FIGURE 10. Possible scenarios explaining our findings. (A) During the first step of biogenesis, the Intimin- and the BamA β-barrel from a hybrid barrel from which the Intimin-barrel buds off into the OM. Then, autotransport of the passenger is initiated and is halted due to the presence of the HA-tag in a hairpin conformation. Direct contact to BamA at this stage is maintained either via interaction of the passenger with POTRA domain(s) or via the folded β-barrels. (B/C) Intimin biogenesis is initiated by the formation of a hybrid barrel with BamA. At this hybrid-barrel stage, the hairpin is inserted. Budding of the Intimin barrel can lead to two outcomes: (B) the passenger stays in the BamA-barrel and mediates contact to the barrel wall or (C) the passenger ends up in the Intimin barrel. Contact to BamA is then mediated either via the POTRA domain(s) or by interaction of the Intimin and the BamA β-barrel domains.

TABLES

| Table 1. Strains and plasmids used in this study. |
|-----------------------------------------------|
| Name       | Relevant genotype or description | Reference |
| E. coli strains |                                      |
| EPEC O127:H6 | enteropathogenic E.coli O127:H6 strain 2348/69 | (50) |
| EPEC O127:H6 ΔeaeA | enteropathogenic E.coli O127:H6 strain 2348/69, ΔeaeA | provided by Prof. Gad Frankel, London, UK; |
| E.c. BL21(DE3)omp2 | BL21(DE3), ompF::Tn5, KanR | (51) |
| Plasmids |                                      |
| pASK-IBA2 | Expression vector with AHTC inducible promoter, AmpR | IBA technologies |
| pASK-IBA2_eaeA | eaeA gene in XbaI-HindIII sites of pASK-IBA2, AmpR | (8) |
| pASK-IBA2_eaeA-HA derivative | Int HA453 in XbaI-HindIII sites of pASK-IBA2 with a tandem HA-tag after residue 453, AmpR | This study |
| pASK-IBA2_eaeA-StrepTag II derivatives | Int wt and Int HA453 in XbaI-HindIII sites of pASK-IBA2 with C-terminal Strep-Tag, AmpR | This study |
Export mechanism of a type Ve autotransporter

Figure 1

A  Int wt

Int wt-Strep

Int HA453

Int HA453-Strep

B  Int wt  Int wt-Strep

Int HA453  Int HA453-Strep

EPEC ΔeaeA  pASK-IBA2
Figure 2

Export mechanism of a type Ve autotransporter
Export mechanism of a type Va autotransporter

Figure 3
Export mechanism of a type Ve autotransporter

Figure 5
Export mechanism of a type Ve autotransporter

Figure 6
Export mechanism of a type Ve autotransporter

Figure 7

[Image showing a Western blot analysis with bands for proteins Intimin, BamA, BamB, BamC, BamD, and BamE, and bands for Skp and SurA under different conditions labeled as Int-wt-Step, Int-HA63-Step, and PASK-IBA2.]
Export mechanism of a type Ve autotransporter

Figure 8
Figure 9
Export mechanism of a type Ve autotransporter

Figure 10
The Inverse Autotransporter Intimin Exports its Passenger Domain via a Hairpin Intermediate

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