The Bam (Omp85) complex is involved in secretion of the autotransporter haemoglobin protease

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Autotransporters are large virulence factors secreted by Gram-negative bacteria. They are synthesized with a C-terminal domain that forms a β-barrel pore in the outer membrane implicated in translocation of the upstream ‘passenger’ domain across the outer membrane. However, recent structural data suggest that the diameter of the β-barrel pore is not sufficient to allow the passage of partly folded structures observed for several autotransporters. Here, we have used a stalled translocation intermediate of the autotransporter Hbp to identify components involved in insertion and translocation of the protein across the outer membrane. At this intermediate stage the β-domain was not inserted and folded as an integral β-barrel in the outer membrane whereas part of the passenger was surface exposed. The intermediate was copurified with the periplasmic chaperone SurA and subunits of the Bam (Omp85) complex that catalyse the insertion and assembly of outer-membrane proteins. The data suggest a critical role for this general machinery in the translocation of autotransporters across the outer membrane.

INTRODUCTION
The autotransporter (AT) pathway is the most widely distributed secretion system used by Gram-negative bacteria to deliver virulence factors across two membranes into the extracellular milieu (Dautin & Bernstein, 2007). The pathway derives its name from the notion that AT proteins, after Sec-dependent transfer to the periplasm, can mediate their own translocation across the outer membrane (OM). AT proteins contain a cleavable signal sequence followed by an N-terminal passenger domain, which contains the effector that is translocated across the OM, and a C-terminal β-domain that is thought to act as a translocase by forming a β-barrel pore in the OM through which the passenger is translocated into the extracellular milieu (Fig. 1a, b). This ‘classical’ view of AT transport has recently been called into question as (i) crystal structures of β-domains (Barnard et al., 2007; Meng et al., 2006; Oomen et al., 2004) and molecular dynamics simulations (Khalid & Sansom, 2006) suggest that the pore formed by the β-domain only allows translocation of extended unfolded protein chains whereas various passengers can be translocated in a partially folded state (Jong et al., 2007; Skillman et al., 2005) and (ii) secretion of several ATs is significantly affected in cells depleted of BamA (or Omp85), the core component of the OM protein (OMP) assembly machinery (Jain & Goldberg, 2007; Voulhoux et al., 2003). Notably, BamA is homologous to the TpsB protein that acts as OM translocase in the ‘two-partner secretion pathway’, a secretion system related to the autotransporter pathway (Jacob-Dubuisson et al., 2009). These findings suggest that an external factor in the OM, possibly the Bam complex, may assist in the translocation of ATs (Bernstein, 2007; Oomen et al., 2004) (Fig. 1c).

We study the translocation of the AT haemoglobin protease (Hbp), a key virulence factor of Escherichia coli EB1 during peritonitis, a severe inflammation of the peritoneal cavity (Otto et al., 2002). After translocation, the Hbp passenger is cleaved by an autocatalytic proteolysis from the β-domain and released as ‘mature’ Hbp into the extracellular milieu, a feature shared by many ATs (Dautin & Bernstein, 2007). Determination of the crystal structure of mature Hbp (Otto et al., 2005) has allowed us to
introduce two unique cysteines into the Hbp passenger that are distant in the primary structure but adjacent in the folded passenger, connecting a long, right-handed β-helical structure to the globular domain that contains a serine protease site (Fig. 1d). This Hbp110C/348C variant was shown to form an intramolecular disulphide bond, catalysed by DsbA, in the periplasm, indicating at least partial folding in the periplasm (Jong et al., 2007). The disulphide bond precluded OM translocation and targeted Hbp110C/348C for degradation by the periplasmic protease DegP. Importantly, in the absence of DegP, Hbp110C/348C accumulated in the OM, with part of the passenger exposed at the cell surface (Jong et al., 2007).

Here, we have further characterized the OM association and folding of the β-domain of this mutant, which appears not to be folded into a heat-modifiable β-barrel and which is not completely inserted into the OM. We have used the Hbp110C/348C stalled translocation intermediate as bait in an unbiased cross-linking and pull-down assay to identify additional components involved in OM insertion and translocation of Hbp. We present evidence that Hbp transiently contacts both the periplasmic chaperone SurA and components of the Bam OMP assembly machinery, and that Hbp translocation depends on both SurA and BamA. Our data provide the first evidence for a direct contact between the Bam machinery and an AT and are consistent with a pivotal role for the Bam machinery in AT translocation.

**METHODS**

**Strains, growth conditions and plasmids.** The E. coli strains used in this study are KS474 (degP:: Km) (Strauch et al., 1989), NR721 (bamB:: Km), NR740 (surA:: Cm) (Ruiz et al., 2005) and JCM166 (Wu et al., 2005), in which bamA is under the control of araBADp. To obtain skp:: Km and ytfM:: Km strains, alleles were transferred using P1 transduction (Silhavy et al., 1984). The Δskp:: Km or ΔytfM:: Km alleles from the Keio collection (Baba et al., 2006) were introduced
into MC4100 to yield MC4100Δxip and MC4100ΔyprM, respectively. The corresponding parental strains were used as a control for Hbp secretion. Strains were grown in M9 medium (Miller, 1992) containing 0.1 % Casamino acids (Difco) and 0.4 % glucose, except for strain JCM166, which was grown in Luria–Bertani medium. When appropriate, kanamycin (30 μg ml\(^{-1}\)) and chloramphenicol (20 μg ml\(^{-1}\)) were added to the medium. Cultures were incubated at 30 °C, except for strains JCM166 and NR740, which were grown at 37 °C. Hbp and its derivatives were expressed from vector pEH3 as described previously (Jong et al., 2007).

Reagents, enzymes and sera. Dithiobis(succinimidylpropionate) (DSP) and disuccinimidyl suberate (DSS) were from Pierce. Other chemicals were from Sigma. Antiserum against the Hbp passenger domain and β-domain were from our own collection. Antiserum against BamA, BamB and SurA were gifts from J. Tommassen (Utrecht University, The Netherlands), T. Silhavy (Princeton University, USA) and R. Koller (Harvard University, USA), respectively. Antiserum against OppA and DnaJ were gifts from K. Igarashi (Chiba University, Japan) and B. Bukau (Heidelberg University, Germany), respectively.

Cross-linking in whole cells and immunopurification. KS474 cells harbouring pEH3-Hbp(-derivatives) were grown to OD\(_{660}\)~0.3 and treated with 50 μM IPTG for 1 h to moderately induce expression. The cells were pelleted (18 000 g, 5 min, 4 °C), washed with buffer (50 mM HEPES pH 8.0, 150 mM NaCl) and resuspended to 15 OD\(_{660}\) units ml\(^{-1}\) in the same buffer. For cross-linking, DSP was added to 2 mM and the samples were incubated on ice for 30 min and quenched with 40 mM glycine. Cross-linked cells were harvested (18 000 g, 5 min, 4 °C), resuspended (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 % NP-40), solubilized with 1 % SDS at 95 °C for 5 min and incubated head-over-head at room temperature for 1 h. Non-solubilized material was removed by centrifugation (9300 g, 10 min). Samples were diluted to 0.1 % SDS and incubated overnight at 4 °C with polyclonal anti-Hbp serum fixed to Protein A Sepharose beads with DSS (Seize X Protein A Immunoprecipitation kit, Pierce). The beads were washed twice with buffer A (10 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.2 % NP-40, 2 mM EDTA), twice with buffer B (10 mM Tris/HCl pH 7.5, 500 mM NaCl, 0.2 % NP-40, 2 mM EDTA) and once with buffer C (10 mM Tris/HCl pH 7.5). Hbp complexes were eluted from the beads with non-reducing sample buffer (50 mM Tris/HCl pH 6.8, 10 % glycerol, 2 % SDS) at 37 °C for 10 min. To dissociate cross-linked products β-mercaptoethanol was added to 5 % and samples were incubated at 37 °C for 15 min followed by 10 min at 95 °C. Samples were analysed using NU-PAGE gradient gels (4–16 %; Invitrogen) followed by silver staining or Western blotting using the indicated antibodies for detection.

In-gel trypsin digestion and mass spectrometry. Selected protein bands from the silver-stained gel (staining compatible with mass spectrometry; Shevchenko et al., 1996) were excised, cut into 1 mm cubes, washed with nanopure water, destained (25 mM ammonium bicarbonate, 60 % acetonitrile, pH 8.5), and then dehydrated (100 % acetonitrile). Shrunken gel pieces were rehydrated (25 mM ammonium bicarbonate), dehydrated again (100 % acetonitrile), and dried in a Speed-Vac. The pellets were resuspended in 8 μl trypsin solution (20 μg ml\(^{-1}\) (Promega) for 1 h, followed by addition of 50 μl 25 mM ammonium bicarbonate buffer to completely immerse the gel pieces. After incubation overnight at room temperature, the samples were purified using a C18 zipstrip and analysed by an Applied Biosystems 4800 MALDI-TOF-TOF. For the MALDI analysis, the samples were spotted using a matrix (7 mg ml\(^{-1}\) α-cyano-4-hydroxycinnamic acid in 10 mM ammonium phosphate, 50 % acetonitrile) and analysed in positive mode. Mass spectra were searched using the Mascot engine against the Swiss-Prot database, release 56.1. For each peptide mass fingerprint search the mass tolerance was set to 0.05 Da. One missed tryptic cleavage was allowed. The mass tolerance for database searching with MS/MS spectra was set to 0.3 Da. All the proteins listed were identified with a confidence interval of 95 % from the MS and/or MS/MS analysis.

Preparation and analysis of outer membranes. KS474 cells harbouring pEH3 constructs were grown to OD\(_{660}\)~0.3 and induced with 50 μM IPTG for 1 h. After French press lysis of ~100 OD\(_{660}\) units of cells, enriched OM fraction was isolated by centrifugation (16 100 g, 15 min, 4 °C). For urea extraction, isolated OMs were resuspended (50 mM Tris/HCl pH 7.8, 150 mM NaCl, 4 M urea), incubated on ice for 30 min and centrifuged (184 000 g, 30 min, 4 °C). The pellet was resuspended in sample buffer without DTT and the supernatant was first TCA precipitated and then resuspended in the same sample buffer. To assess heat modifiability, isolated OMs were resuspended in sample buffer (minus DTT) and incubated for 10 min at room temperature, 37 °C or 95 °C. Samples were analysed by SDS-PAGE and Western blotting using antiserum against the β-domain for detection.

RESULTS

The Hbp110C/348C translocation intermediate is adjacent to SurA and the Bam complex

Passenger translocation is a very rapid process, meaning that putative contacts of the unprocessed AT (pro-Hbp) with external factors aiding in translocation are short-lived (Skillman et al., 2005). To improve the chances of identifying protein contacts between pro-Hbp and additional factors we made use of the paired cysteine mutant Hbp110C/348C. We propose Hbp110C/348C to represent a translocation intermediate that is stalled during translocation across the OM due to the incompatibility of a disulphide bond between domains of the passenger and transfer across a relatively narrow channel. Sedimentation gradient analysis and differential detergent solubilization have shown that Hbp110C/348C cofractionates with the OM mainly as pro-Hbp (Jong et al., 2007). To further confirm that Hbp110C/348C is located in the OM rather than forming aggregates in the periplasm, we performed flotation gradient analysis to show that Hbp110C/348C floats to the same position as the OM porins (Supplementary Fig. S1). Our previous whole-cell immunofluorescence data have also shown that Hbp110C/348C is in part surface exposed (Jong et al., 2007). To enable detection by immunofluorescence, Hbp110C/348C was expressed at a relatively high level upon induction with 200 μM IPTG. To rule out that expression of Hbp110C/348C at this level had affected the integrity of the OM we now confirmed that antibodies have no access to the periplasm under the conditions used (Supplementary Fig. S2), providing further evidence that Hbp110C/348C is a translocation intermediate. Notably, the immunofluorescence intensity of the accumulated Hbp110C/348C under these conditions suggests that most Hbp110C/348C molecules are surface exposed, probably stalled at the same insertion stage (see supplementary data for details).
In the present study we expressed Hbp110C/348C at a lower level (50 μM IPTG) to reduce saturation of putative partner proteins and possible expression-related stress. Expression at this level did not affect cell viability. Notably, under these conditions a considerable fraction (~40%) of Hbp110C/348C is processed to mature Hbp (see Fig. 2a, lane 3), which is secreted. Most likely, this is due to kinetic partitioning between disulphide bond formation and translocation. The Hbp110C/348C intermediate and putative interacting components were purified using antibodies against the Hbp passenger domain that were covalently linked to Protein A Sepharose beads (anti-Hbp-beads). Initial attempts using isolated OM fractions did not reveal any interacting proteins, possibly due to the transient nature of these interactions (data not shown). Therefore, protein–protein interactions were stabilized in whole cells using chemical cross-linking to preserve the original contacts. The homobifunctional, lysine-specific cross-linker DSP was used because it is membrane-permeable and thiol-cleavable to permit release and analysis of cross-linked partners. After cross-linking, crude membrane fractions were isolated, solubilized and subjected to immuno-affinity purification. Hbp and cross-linked partners were released from the anti-Hbp-beads using sample buffer and cross-linking was reversed using β-mercaptoethanol. As controls, cells not expressing Hbp, or cells expressing wild-type Hbp or a Hbp variant that is efficiently translocated but not processed after translocation (Δβ-cleavage) were used (Jong et al., 2007). Analysis of the eluates by SDS-PAGE and silver staining (Fig. 2a) showed that Hbp and its derivatives were efficiently purified. Using Hbp110C/348C, the level of the pro-Hbp form was reduced upon DSP treatment, suggesting that it had been efficiently cross-linked (compare lanes 3 and 7). It should be noted that samples were treated with β-mercaptoethanol to dissociate the cross-linked products but that this step is not completely efficient. Four proteins specifically copurified with cross-linked Hbp110C/348C (compare lanes 3 and 7). These proteins were identified by mass spectrometry as: (a) BamA (formerly designated YaeT, also known as Omp85 in Neisseria meningitidis), (b) SurA, (c) BamB (or YfgL) and (d) OmpA (Supplementary Table S1). Band (e) was found to be a degradation product of Hbp (data not shown). Western blotting confirmed the identity of the partners (Fig. 2b, lane 7), and showed that significant enrichment of the partners depended on the use of Hbp110C/348C. The presence of small amounts of BamA and SurA in the eluates from cross-linked wild-type Hbp and the Δβ-cleavage mutant (lanes 6 and 8) may be attributed to a small population of 'natural intermediates', i.e. Hbp passengers that are in the process of being translocated. Importantly, these controls suggest that the stalled pro-Hbp110C/348C intermediate, rather than the cleaved mature Hbp110C/348C 'contamination' or fully translocated pro-Hbp110C/348C, is in the proximity of SurA, BamA, BamB and OmpA.

Interestingly, BamA and BamB participate in a recently identified machinery that is involved in the insertion of OMPs (Bos et al., 2007; Ruiz et al., 2006). OmpA is an abundant OMP (~300,000 copies per cell (Movva et al., 1980) with a large periplasmic domain that is similar to the C-terminal domain of RmpM from N. meningitidis, a protein described to be associated with the Bam machinery.

![Fig. 2](http://mic.sgmjournals.org/3985)

A translocation intermediate of the AT Hbp, Hbp110C/348C, is cross-linked to SurA, BamA, BamB and OmpA. (a) Silver-stained SDS-polyacrylamide gel of samples of cross-linked cells after immuno-purification with anti-Hbp. Cells expressing the indicated Hbp derivatives were incubated with DSP (lanes 5–8) or mock treated (lanes 1–4). Crude membrane fractions were isolated, solubilized and immuno-purified with anti-Hbp beads. Eluates were treated with β-mercaptoethanol to cleave DSP prior to electrophoretic analysis. Protein bands [a–e] were identified by mass spectroscopy: a, BamA; b, SurA; c, BamB; d, OmpA; e, Hbp degradation product. The asterisks indicate antibody contamination. Pro-Hbp and mature Hbp are annotated as p and m, respectively. (b) Western blot of samples obtained in (a) using the indicated antibodies.
in this species (Bos et al., 2007). A role of RmpM or OmpA in OMP biogenesis has not been reported. SurA is a periplasmic chaperone thought to target a subset of OMPs to the Bam complex (Sklar et al., 2007). Together, the results indicate that the stalled Hbp is in contact with SurA and the Bam complex. These interactions probably occur only during translocation of the Hbp passenger, as they are much less prominent when translocation is complete (wild-type Hbp), even when the passenger is not cleaved from the \( \beta \)-domain (\( \Delta^-\beta \)-cleavage mutant).

To rule out that the observed cross-linked adducts are specific for the Hbp110C/348C intermediate, we also analysed the cross-linked partners of another Hbp mutant stalled during translocation across the OM. In this mutant, Hbp-Calm, which has already been described and characterized (Jong et al., 2007), domain 2 of Hbp (Fig. 1d) has been replaced by calmodulin. Hbp-Calm folds into a translocation-incompetent structure in the presence of \( Ca^{2+} \) but in its absence the intermediate condition can be reverted (Jong et al., 2007). Hbp-Calm resulted in the same cross-linking pattern as the Hbp110C/348C mutant, suggesting that the contacts of stalled pro-Hbp with BamA, BamB, SurA and OmpA are generic (Supplementary Fig. S3).

**The \( \beta \)-domain of Hbp110C/348C is not fully inserted and folded into the OM**

Previously, we established that Hbp110C/348C can be detected on the cell surface using antibodies that detect the Hbp passenger domain (Jong et al., 2007) (Supplementary Fig. S2). According to the classical AT model this would imply that the passenger domain is stuck during translocation in a fully assembled \( \beta \)-barrel formed by the C-terminal domain. This is difficult to reconcile with the cross-links to SurA and BamA. BamA is thought to transiently associate with nascent OMPs delivered by SurA or Skp to mediate their insertion and folding (Bos et al., 2007; Ruiz et al., 2006). The periplasmic POTRA domains in BamA have been implicated in recognition of the C-terminal signature OMP motif, which is also present in Hbp, and nascent \( \beta \)-strands of OMPs (Robert et al., 2006). Hence, the cross-links to SurA and BamA suggest that the C-terminal \( \beta \)-domain is not yet fully folded and inserted into the OM. Therefore, we decided to examine the nature of OM association of the Hbp110C/348C intermediate by urea extraction, which enables discrimination between proteins that are fully inserted into the OM and proteins that are associated with, but not inserted, into the OM (Collin et al., 2007; Voulhoux et al., 2003).

Enriched OM fractions containing wild-type Hbp, Hbp110C/348C or the \( \Delta^-\beta \)-cleavage mutant were incubated in 4 M urea and subjected to ultracentrifugation. Pellet and supernatant fractions were analysed by SDS-PAGE and Western blotting using \( \beta \)-domain-specific antibodies. Only the Hbp110C/348C intermediate that accumulates as pro-Hbp was significantly extracted from the OM (Fig. 3a, lanes 4–6), suggesting that it is not fully integrated in the membrane at this stage in biogenesis. In contrast, the cleaved \( \beta \)-domain of wild-type Hbp (Fig. 3a, lanes 1–3), as well as the \( \Delta^-\beta \)-cleavage mutant, which contains a functionally assembled \( \beta \)-domain and a surface-exposed passenger domain (Fig. 3a, lanes 7–9), were fully resistant to extraction. The urea-resistant, cleaved \( \beta \)-domain detected for Hbp110C/348C originates from molecules that have escaped disulphide bond formation and stalling (described above; Fig. 3a, lanes 4–6). Overall the results suggest that the \( \beta \)-domain of the stalled Hbp translocation intermediate is not properly integrated in the membrane.

Extraction of pro-Hbp110C/348C from the OM by urea treatment suggests that the \( \beta \)-domain of the stalled translocation intermediate is not yet folded into a \( \beta \)-barrel conformation. To investigate this possibility we examined the so-called heat modifiability of this protein. Correctly folded \( \beta \)-barrels retain their conformation upon solubilization in SDS and are only completely denatured upon

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**Fig. 3.** The \( \beta \)-domain of Hbp110C/348C is not folded and not fully inserted in the OM. (a) Western blot of urea-treated OM preparations from cells expressing the indicated Hbp derivatives, using anti-\( \beta \)-domain serum for detection. Extracted material (S) was separated from the integral membrane fraction (P) by ultracentrifugation; input material was included for reference (T). (b) Western blot of enriched OM fractions with heat-modified Hbp derivatives using anti-\( \beta \)-domain serum for detection. OMs were solubilized in sample buffer and incubated at room temperature (RT), 37 °C or 95 °C for 10 min. Unfolded (u-) and folded (f-) forms of pro-Hbp and \( \beta \)-domain are indicated.
heating, resulting in different electrophoretic mobilities for solubilized and heat-denatured β-barrels. Enriched OM fractions containing Hbp derivatives were dissolved in sample buffer at room temperature, 37 °C or 95 °C for 10 min and analysed by SDS-PAGE and Western blotting using β-domain-specific antibodies. Heat modifiability was observed for the cleaved β-domain of wild-type Hbp, the cleaved β-domain of the fraction of Hbp110C/348C that escaped trapping, and the Δ−β-cleavage pro-Hbp, indicating properly folded β-barrels for all these proteins (Fig. 3b). In contrast, the accumulated pro-Hbp110C/348C showed heat-insensitive migration, indicating that its β-domain had not yet folded into a stable β-barrel conformation.

Together, the data show that the stalled Hbp110C/348C translocation intermediate has a β-domain that has not folded into a β-barrel and that is not completely inserted into the OM.

### BamA and SurA are required for secretion of Hbp

To investigate the functional relevance of the transient proximity of BamA, BamB and SurA in Hbp biogenesis, we analysed Hbp expression and secretion in strains either depleted for BamA or deficient in BamB or SurA. BamA is an essential integral membrane protein in the Bam complex and therefore considered to be the key element of the insertase. Wild-type Hbp was expressed in a strain in which the sole copy of\textit{bamA} is under\textit{araBAD}\_p control (Wu et al., 2005). In this strain, BamA is depleted by removing arabinose from the culture medium. After 3 h of depletion, Hbp expression was induced with 1 mM IPTG for 1 h at 37 °C. At this point, the BamA level was reduced to ~5 % of the level in the parental strain grown under the same conditions (Fig. 4a, compare lanes 1 and 5). Under these conditions pro-Hbp accumulated in the cells and mature Hbp was not detected in either the culture medium or the cell fraction. Even in the presence of L-arabinose inducer, processing of Hbp was slightly reduced as compared to the control parental strain, which may reflect the somewhat lower BamA level in the complemented cells (Fig. 4a, compare lanes 3–4 and 7–8). Depletion of BamA affected the growth rate over time, although after 1 h Hbp was not detected in either the culture medium or the cell fraction.
induction (4 h BamA depletion) cells were still viable with an intact OM as judged by the absence of the intracellular marker protein DnaJ and the periplasmic marker protein OppA in the medium fraction (Fig. 4a, lanes 5 and 6). YtfM, a second, non-essential member of the Omp85 family in E. coli (Stegmeier et al., 2007), appeared dispensable for Hbp biogenesis and secretion (Supplementary Fig. S4). Hence, the data strongly suggest that BamA is required for secretion of Hbp.

Although BamB is nonessential, it is highly conserved and the levels of many OMPs are reduced when BamB is absent (Charlson et al., 2006; Ruiz et al., 2005). When Hbp was expressed in the bamB knockout strain, growth was not impaired but Hbp was processed and secreted to levels almost identical to the parental strain (Fig. 4b, top panel). It should be noted that expression, processing and extracellular release of mature Hbp is somewhat strain-dependent (compare the top panels in Fig. 4). Importantly, the cleaved \( \beta \)-domain accumulated in a heat-modifiable form in bamB knockout cells, demonstrating that BamB is not required for stability and folding of the \( \beta \)-domain (Fig. 4b, second panel). We verified that the bamB deletion was complete and there was no significant cell lysis upon Hbp expression using DnaJ as a cytosolic marker protein. Furthermore, the integrity of the OM appeared uncompromised since the periplasmic marker OppA was not detected in the medium fraction (Fig. 4b, lower panels).

SurA is a periplasmic chaperone that delivers proteins to the Bam complex via a direct association with BamA (Sklar et al., 2007). Under most growth conditions, SurA is not essential but cells that lack SurA contain reduced levels of OMPs and the permeability of their OM is compromised. Hbp expressed in a surA knockout strain accumulated in the uncleaved pro-form (Fig. 4c, lane 3) and impaired cell growth. The minor fraction of mature Hbp formed was efficiently released from the cell surface into the culture medium whereas the cleaved \( \beta \)-domain and the cytosolic reporter DnaJ were retained in the cells (Fig. 4c, lanes 3 and 4). The OM appeared somewhat leaky for the periplasmic marker OppA but the accumulated pro-Hbp was still retained in the cell fraction as in the case of BamA depletion. The majority of mature Hbp appeared to be released into the medium in the absence of SurA. However, since the mechanism of release of cleaved passenger from the cells is not understood, the basis of the influence of SurA on this late step remains obscure. The general periplasmic chaperones Skp and DegP have also been implicated in targeting of OMPs to the Bam complex, operating in a pathway that runs parallel to the main SurA pathway (Bos et al., 2007; Sklar et al., 2007). Our data indicate that both DegP and Skp are dispensable for Hbp biogenesis and secretion (Jong et al., 2007; Supplementary Fig. S4) and are also unable to rescue the escort of off-pathway Hbp produced in the absence of SurA (Fig. 4c).

Combined, the data suggest an important role for SurA and BamA in Hbp biogenesis and secretion consistent with their proximity to the Hbp110C/348C translocation intermediate.

DISCUSSION

Recent studies question the classical view of autotransport as a self-sufficient secretion system in which the C-terminal domain of the AT (the \( \beta \)-domain) functions as a pore in the OM to allow the passage of the N-terminal passenger domain of the AT (Bernstein, 2007). Essentially, crystal structures of the \( \beta \)-domains of two ATs revealed a narrow 12-strand \( \beta \)-barrel pore structure with an inner diameter of \( \sim 1.2 \) nm (Barnard et al., 2007; Oomen et al., 2004) that is incompatible with the reported secretion of small folded elements and closely spaced cysteine bridges engineered or naturally occurring in AT passenger domains (Barnard et al., 2007; Bodelon et al., 2009; Brandon & Goldberg, 2001; Jong et al., 2007; Oomen et al., 2004; Skillman et al., 2005). These incongruent data have led to discussions about the possible involvement of host factors in the OM translocation of ATs (Bernstein, 2007; Oomen et al., 2004).

To identify host factors that are potentially involved in OM translocation of ATs we used chemical cross-linking to analyse the molecular environment of a paired cysteine mutant of the AT Hbp, Hbp110C/348C, that appears to be stalled during translocation across the OM (Jong et al., 2007). Disulphide bond formation in Hbp110C/348C was shown to occur in the periplasm, catalysed by DsbA, creating an Hbp variant that is exclusively localized in the OM (Jong et al., 2007) (Supplementary Fig. S3). Most likely, Hbp110C/348C represents a translocation intermediate as it is partly exposed to both the periplasm (judged by its sensitivity to the periplasmic protease DegP), and the cell surface (judged by whole-cell immunofluorescence and blotting; Jong et al., 2007) (Supplementary Fig. S2 and data not shown). Of note, the same paired cysteine approach was recently used to successfully prepare an OM translocation intermediate of the AT pertactin from Bordetella pertussis (Junker et al., 2009).

Surprisingly, we find that Hbp110C/348C can be extracted from the membrane with urea, suggesting that its \( \beta \)-domain is in a proteaceous environment rather than being embedded in the OM lipids (Fig. 3a). Furthermore, Hbp110C/348C is not heat-modifiable, indicating that it is not yet folded into a \( \beta \)-barrel structure (Fig. 3b). These data are difficult to reconcile with a simple model in which the surface-exposed Hbp110C/348C passenger is stalled in its own adjacent \( \beta \)-domain pore.

When Hbp110C/348C was used as a bait in an unbiased pull-down approach upon whole-cell cross-linking, four proteins were copurified: BamA, BamB, SurA and OmpA. BamA, BamB and SurA participate in a recently identified conserved machinery that plays a generic role in the targeting and assembly of OM proteins (Voulhoux et al., 2003; Wu et al., 2005). Importantly, these proteins were hardly copurified with wild-type Hbp or a fully translo-
icated but unprocessed Hbp derivative that is still associated with the OM. This strongly suggests that the co-purified components reflect an interaction of Hbp with the Bam machinery upon its targeting to the OM, during translocation of the passenger domain. Apparently, these interactions dissociate when translocation is complete. The transient nature of such contacts probably explains the reported inability to find interacting partners of fully assembled ATs in the OM (Muller et al., 2005; Skillman et al., 2005).

What could be the role of the Bam complex in Hbp secretion? How can we reconcile the proximity of Hbp110C/348C to the Bam complex with its partial translocation and with the fact that its β-domain is not properly folded and integrated in the OM? Possibly, BamA acts as the channel for vectorial transport of the Hbp passenger before its β-domain is fully assembled in the OM. Intriguingly, the 16-stranded β-barrel at the C-terminus of BamA is thought to constitute a pore structure (reviewed by Bos et al., 2007). Liposome swelling data indicate that BamA forms a pore of ~2.5 nm which could be compatible with the observed translocation of small folded elements or partially folded structures in ATs (Robert et al., 2006) but might be too narrow for translocation of the partially folded and disulphide-bonded N-terminal region of the Hbp110C/348C passenger. The pore-function of BamA appears to be evolutionarily conserved and is also found in the homologous protein pores Toc75 in the plastid OM and TpsB in the bacterial OM, which are both homologous to BamA (Bos et al., 2007). In this scenario, the role of the β-domain might be restricted to delivery of pro-Hbp at the Bam complex. The C-terminal motif of OMPs is known to influence BamA pore activity (Robert et al., 2006) via an interaction between its C-terminal OMP signature motif and the N-terminal POTRA domains of BamA. By analogy the interaction of the AT C-terminal motif could trigger increased pore activity of BamA that might allow passenger translocation (Robert et al., 2006). Subsequently, the β-domain would integrate into the OM upon folding into a β-barrel structure and function in temporary anchoring and subsequent processing of the translocated Hbp passenger (Dautin et al., 2007).

Another explanation for the combined data is that the Bam complex coordinates both passenger translocation and β-domain assembly in a concerted mechanism. In this model, the passenger could still be translocated through the β-domain that is accommodated in the Bam pore in an extended non-native conformation (Bernstein, 2007). In this scenario, it is difficult to envision the final partitioning of the β-domain into the OM lipids because it would imply a lateral opening of the Bam channel, which is thermodynamically highly unfavourable for a β-barrel structure. In fact, in both the channel and concerted model the later stages of translocation such as the dissociation of Hbp from BamA are difficult to reconcile with a monomeric BamA. Interestingly, BamA and BamA-like proteins have been shown to oligomerize in vitro (Paschen et al., 2003; Robert et al., 2006; Surana et al., 2004), suggesting the existence of a hydrophobic central cavity as an insertion site for OMPs docked to the Bam complex by SurA. The binding of SurA could then trigger conformational changes of the Bam complex to accommodate an ‘expanded’, loosely folded β-barrel in that hydrophobic core. In this model, the expanded β-domain of Hbp would translocate the passenger domain while surrounded by the BamA oligomer. An expanded β-domain could be compatible with the observed translocation of small folded elements (Jong et al., 2007; Skillman et al., 2005). When translocation of the passenger is completed the Bam complex would disassemble, which might trigger the sealing of the β-domain and its folding into a stable barrel permitting the cleavage of the passenger domain. Our data are consistent with this oligomeric model since the expanded β-domain of Hbp110C/348C trapped in the Bam oligomer is expected to be extractable with urea and might not yet be folded in a heat-modifiable structure.

BamB is one of three accessory lipoproteins in the Bam complex of which the function is not known. Considering its relatively low abundance in the eluates and the fact that BamB did not appear to be required for Hbp secretion, its copurification with the Hbp110C/348C intermediate may reflect secondary cross-linking to BamA rather than a direct interaction with the intermediate itself. In any case, a close proximity of BamB is likely, since a direct interaction between BamA and BamB has been demonstrated recently (Vuong et al., 2008). The copurification of OmpA is more enigmatic since it is not a known constituent of the Bam complex. Intriguingly, however, RmpM, the Neisseria meningitidis homologue of OmpA, has been identified as a component of the Omp85 complex but a function in OMP assembly has not been reported (Bos et al., 2007). Similar to BamB, OmpA is not required for secretion of Hbp (Supplementary Fig. S4). Copurification of OmpA may also be due to a delay in its membrane assembly near Bam complexes that are occupied by the Hbp110C/348C intermediate. The high abundance of OmpA and the fact that it contains a large periplasmic domain may contribute to its rather selective copurification.

Involvement of the Bam complex in the biogenesis of ATs is consistent with recent genetic studies showing that processing and secretion of several ATs, including Hbp, is significantly affected in cells depleted of the central pore-forming BamA subunit (Bernstein, 2007; Bodelon et al., 2009; Bos et al., 2007; Jain & Goldberg, 2007; this study, Fig. 4). However, since BamA is essential for the assembly of many OMPs (Voulhoux et al., 2003; Wu et al., 2005), it is difficult to discriminate in these studies between a role of BamA as an insertase for the AT β-domain (or for an independent host-derived translocator) versus a more direct role in translocation of the AT passenger domain.

Little is known about the role of periplasmic chaperones and folding catalysts in autotransport. Genetic analysis revealed that DegP, Skp and SurA are directly or indirectly
required for proper surface presentation of IcsA in *Shigella flexneri* (Purdy et al., 2007). Our combined genetic and biochemical data indicate a primary role for SurA in biogenesis of Hbp rather than crucial functions for DegP, DsbA (Jong et al., 2007) and Skp (Supplementary Fig. S4). The copurification of SurA with Hbp110C/348C provides the first biochemical evidence for its role in the delivery of OMP assembly intermediates at the Bam complex, consistent with its observed cross-linking to the Bam complex (Sklar et al., 2007). Binding of SurA to Hbp may already occur at the inner membrane facilitated by the transient anchoring of Hbp at the inner membrane via its unusual signal peptide (Szabady et al., 2005). It remains to be determined whether SurA binds to the passenger domain, to the β-domain [analogous to the interaction of SurA with non-native OMPs (Behrens et al., 2001; Ureta et al., 2007)], or to both.

In conclusion, our data are consistent with a role of the Bam complex and SurA in OM targeting and translocation of the Hbp passenger domain. The exact role of the Bam complex in Hbp biogenesis awaits further analysis. Conceivably, the fundamental mechanisms of Bam-mediated membrane integration of OMPs (which often contain considerable extracellular loops) and ATs are related. Further analysis of the Hbp110C/348C/SurA/Bam complex and other Hbp translocation intermediates may therefore provide insight into the basic function of the Bam complex.

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