Signal Recognition Particle (SRP)-mediated Targeting and Sec-dependent Translocation of an Extracellular *Escherichia coli* Protein*

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Hemoglobin protease (Hbp) is a hemoglobin-degrading protein that is secreted by a human pathogenic *Escherichia coli* strain via the autotransporter mechanism. Little is known about the earliest steps in autotransporter secretion, *i.e.* the targeting to and translocation across the inner membrane. Here, we present evidence that Hbp interacts with the signal recognition particle (SRP) and the Sec-translocon early during biogenesis. Furthermore, Hbp requires a functional SRP targeting pathway and Sec-translocon for optimal translocation across the inner membrane. SecB is not required for targeting of Hbp but can compensate to some extent for the lack of SRP. Hbp is synthesized with an unusually long signal peptide that is remarkably conserved among a subset of autotransporters. We propose that these autotransporters preferentially use the co-translational SRP/Sec route to avoid adverse effects of the exposure of their mature domains in the cytoplasm.

Hemoglobin protease (Hbp)[1] is secreted by a human pathogenic *Escherichia coli* strain (1) and contributes to the pathogenic synergy between *E. coli* and *Bacteroides fragilis* in intraabdominal infections (2). It represents the first described member of the serine protease autotransporters of Enterobacteriaceae (SPATE) group of autotransporter proteins (3).

The key feature of an autotransporter is that it contains all the information for secretion in the precursor of the secreted protein itself (3). Autotransporters comprise three functional domains: 1) an N-terminal targeting domain; 2) a C-terminal translocation domain; and, in between these two, 3) the passenger domain that is the actual secreted moiety. The C-terminal domain is supposed to form a β-barrel structure in the outer membrane that may form an oligomeric channel around a cavity to allow the passage of the passenger domain (4). The N-terminal domain is thought to function as a signal peptide to mediate targeting to and translocation across the inner membrane.

Compared with other signal peptides in *E. coli*, the putative signal peptide of Hbp is unusually long (1) (Fig. 1). Analogously, several other autotransporters are predicted to have long signal peptides (3, 5). All these signal peptides display a conserved domain structure. The C terminus resembles a normal signal peptide with a basic N-terminal region, a hydrophobic core region, and a C-terminal consensus signal peptidase cleavage site. The N terminus forms a conserved extension, the function of which is not known (Fig. 1).

Most periplasmic and outer membrane proteins synthesized with a cleavable signal peptide are translocated through the Sec-translocon. The core translocase consists of the integral inner membrane proteins (IMPs) SecY, SecE, and SecG, which constitute an oligomeric complex homologous to the Sec61 channel complex in the endoplasmic reticulum (6). The peripheral membrane ATPase SecA is unique to bacteria and catalyzes the actual polypeptide transfer through the translocase. Targeting to the Sec-translocon may occur after translation and often requires the cytosolic chaperone SecB.

The Sec-translocon is also used for the membrane insertion of most IMPs that are synthesized with uncleaved, relatively hydrophobic signal peptides (7). Targeting of IMPs to the Sec-translocon is not mediated by SecB but by the signal recognition particle (SRP) and its receptor, FtsY, in a co-translational mechanism that resembles targeting to the Sec61 complex in the endoplasmic reticulum (8).

Here we provide evidence that the long signal peptide of Hbp mediates targeting to the inner membrane via the SRP pathway. When the SRP pathway is compromised, SecB can prevent, to a certain extent, the mislocalization of pre-pro-Hbp. Subsequent translocation across the inner membrane involves the Sec-translocon. This is the first demonstration of the use of the co-translational SRP pathway for inner membrane targeting of an extracellular protein.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Media—*E. coli* K12-strains and the plasmids used are listed in Table I. *E. coli* strains were routinely grown in Luria-Bertani (LB) medium (9). Strains MM152 and HDB52 were grown in M9 medium (9). If required, antibiotics were added to the culture medium.

Reagents and Sera—Restriction enzymes, the Expand long template PCR system and the Lumi-light Western blotting substrate were obtained from Roche Molecular Biochemicals. All other chemicals were supplied by Sigma. Antiserum J40 raised against purified Hbp has been described previously (2). Antisera against β-lactamase, OmpA/OmpC, trigger factor (TF)/SecA, and SufI were gifts from J.-M. van Dijl, J.-W. de Gier, W. Wickner, and T. Palmer, respectively.

Plasmid Construction—For cross-linking of nascent Hbp, we constructed pC4MethssHbp, which encodes the 110 N-terminal amino acid residues of Hbp fused to a C-terminal 4× methionine tag and a three amino acid linker sequence to improve labeling efficiency. The construct

[1] The abbreviations used are: Hbp, hemoglobin protease; IMP, inner membrane protein; IMV, inverted membrane vesicle; SRP, signal recognition particle; DSS, 2,2-dimethyl-2-silapentanesulfonic acid; TF, trigger factor; TPS, two-partner secretion.
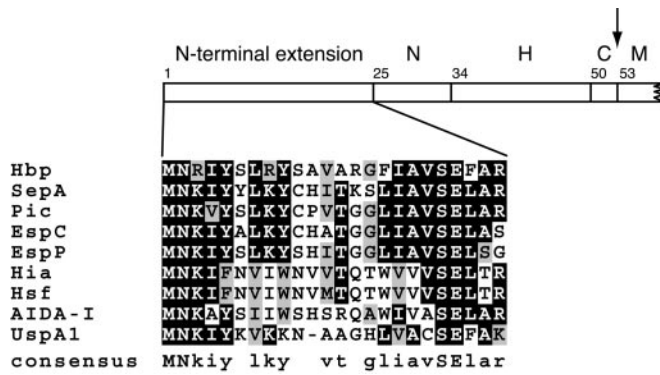
FIG. 1. **The signal peptide of Hbp has a conserved N-terminal extension.** A schematic representation of the 52 amino acid-long signal peptide of Hbp is shown, indicating the probable signal peptidase cleavage site (*arrow*). The basic N-terminal (*N*), hydrophobic (*H*), and C-terminal (*C*) domains characteristic of typical signal peptides are indicated. *M* indicates the beginning of the mature region of Hbp. A comparison of the N-terminal extensions of several autotransporters possessing extended signal peptides is given together with a consensus sequence of the conserved domain.

was obtained by PCR using pHE12.6 as a template and the primers Hbp-*Eco*RI-for (5′-GCCG**GAATTC**TAATATGAACAGAATTTATTCTC-TTC-3′ with the *Eco*RI restriction site in boldface) and Hbp-*Bam*HI-rev (5′-GCG**GGATCC**ACCGATTTCCGAATCCACA-3′ with the *Bam*HI restriction site in boldface). The PCR fragment was cloned *Eco*RI/ *Bam*HI into pC4Meth (10). To construct plasmid pHB6.4-HbpΔss, the signal peptide coding region of *hbp* was removed from plasmid pHB6.4-Hbp using the Exsite PCR-based, site-directed mutagenesis kit (Stratagene). The plasmid pHB6.4-Hbp is derived from pHE12.6 (1). The primers used were Hbp-*Nhe*I-for (5′-CTT**GCTAGC**GTCAATA-ATGAA-CTCGGGTATC-3′ with the *Nhe*I restriction site in boldface) and Hbp-*Bgl*II-rev (5′-CTGATTTTATTTTTCTCAGGAGTAATTAAAAATGAAG-**AGATCT**AAG-3′ with the *Bgl*II restriction site in boldface). Upon ligation of the linear DNA, the last three bases from each end constitute a *Hin*dIII restriction site. The resulting plasmid encodes the Hbp protein without its signal peptide but with six extra N-terminal amino acids (MKRSKL) and two altered amino acids (GT → AS) at the start of the mature Hbp region.

In Vitro Transcription, Translation, Targeting, and Cross-linking—Truncated mRNA was prepared as described previously (11) from *Hin*dIII linearized pC4MethssHbp. *In vitro* translation, targeting to inverted membrane vesicles (IMVs), cross-linking with DSS, and carbonate extraction of nascent Hbp were carried out as described (11). The samples were either analyzed directly by 15% SDS-PAGE or immunoprecipitated first using 4-fold the amount used for direct analysis.

Pulse-Chase Experiments—For Ffh depletion studies, strains HDB51 and HDB52 were grown overnight in M9 containing 0.1% casaminoacids (Difco), 0.2% fructose, and 0.2% L-arabinose, washed in the same medium lacking L-arabinose, and diluted to an OD$_{660}$ of 0.004 in M9 containing fructose (0.2%) and either L-arabinose (0.2%) or glucose (0.2%). Cells were grown to an OD$_{660}$ of ~0.3 before labeling. Depletion of Ffh was verified by Western blotting. The temperature-sensitive SecY mutant strains IQ85 and its isogenic wild type strain IQ86 were grown overnight at 30 °C in LB medium, diluted into fresh medium to an OD$_{660}$ of 0.02, and grown to an OD$_{660}$ of 0.3. Growth was then continued at 30 or 42 °C for 3 h. For overexpression of a dominant lethal *ftsY* allele, strain BL21(DE3) harboring pET9-FtsY-A449 was grown overnight at 37 °C in M9 medium with 0.2% glycerol as a carbon source and diluted into fresh medium to an OD$_{660}$ of 0.03. When cells reached an OD$_{660}$ of 0.2, 40 μM isopropyl-1-thio-β-D-galactopyranoside was added to induce FtsY-A449 expression, and growth was continued for 15 min. To inhibit SecA functioning in MC4100, 3 mM NaN$_3$ was added 3 min prior to labeling. In all experiments, 2.4 OD$_{660}$ units of cells were washed and diluted into 3 ml of M9 medium containing appropriate sugars and a mixture of 18 amino acids except methionine and cysteine. After recovery for 15–45 min at 30, 37, or 42 °C as indicated, cells were pulse labeled for 1 min by the addition of 10 μCi/ml [^{35}S]methionine and chased for various times by adding cold methionine (2 mM). To stop the chase, cells were rapidly cooled in ice water and centrifuged at 4 °C for 2 min at 8,000 × *g*. Supernatants were precipitated with trichloroacetic acid and subjected directly to 8–15% SDS-PAGE. Cell pellets were first lysed and subjected to immunoprecipitation using anti-Hbp and anti-

OmpA serum essentially as described (12).

Western Blotting—For analysis of steady-state levels of Hbp, *E. coli* strains harboring an *hbp* expression plasmid were grown to an OD$_{660}$ of 0.5. Aliquots were removed from the cultures and centrifuged (1 min at 16,000 × *g*). The culture supernatants were trichloroacetic acid precipitated. Equivalent amounts of cells and supernatant were analyzed by Western blotting. Blots were developed by enhanced chemiluminescence using Lumi-light Western blotting substrate.

Sample Analysis—Radiolabeled proteins were visualized by phosphorimaging using a Amersham Biosciences PhosphorImager 473 and quantified using the ImageQuant quantification software from Amersham Biosciences. Chemiluminescent Western blots were analyzed using the Fluor-S MultiImager and Multianalyst software (Bio-Rad).

RESULTS

Hbp That Lacks Its Signal Peptide Is Not Secreted and Degraded in the Cytosol—Hbp is synthesized in a pre-pro form (148 kDa) (1). The N-terminal signal peptide is cleaved during passage of the pre-pro-Hbp through the inner membrane, leaving the pro-Hbp (142 kDa) in the periplasm. The C-terminal β-barrel domain is cleaved from the pro-Hbp at the outer membrane and mediates the transfer of the mature Hbp (111 kDa) into the culture medium. To analyze the role of the Hbp signal peptide, it was deleted from the pre-pro-Hbp, and the consequences for maturation and secretion were analyzed by Western blotting of cell samples and culture supernatants using Hbp-specific antibodies (Fig. 2*A*). As expected, deletion of the signal peptide (Δss) prevented secretion of mature Hbp into the medium. Only a small amount of pro-HbpΔss was detected in the cells. In contrast, most wild-type pre-pro-Hbp was processed to mature Hbp, which was either secreted or remained cell-associated as observed previously (1). In addition, some pre-pro- or pro-Hbp accumulated in the cells.

We next investigated the reason for the low expression level of pro-HbpΔss. It has been observed previously that secreted proteins and IMPs that fail to be translocated across or inserted into the inner membrane are prone to proteolytic degradation (13). To investigate this possibility, HbpΔss was expressed in strain HDB107, which lacks the major cytoplasmic proteases Lon and ClpYQ (13). Pulse-chase labeling was employed to compare the stability of HbpΔss species in strain HDB107 and its isogenic parental strain HDB97 (Fig. 2*B*). HbpΔss remained almost completely stable for at least 10 min in HDB107, whereas only limited amounts were detected in HDB97 after a 10-min chase. In fact, relatively little HbpΔss was detected in HDB97 even when samples were analyzed directly after pulse labeling.

These data indicated that Hbp requires its signal peptide for targeting to the inner membrane and, consequently, for secretion of mature Hbp. Mislocalized pro-Hbp is rapidly degraded by the cytoplasmic proteases Lon and/or ClpYQ.

Nascent Hbp Interacts with SRP, Trigger Factor, SecA, and SecY in Vitro—To investigate the molecular interactions of the atypical Hbp signal peptide in the cytoplasm and the membrane, we used an *in vitro* cross-linking assay. A radiolabeled Hbp translation intermediate of 117 amino acid residues was generated by *in vitro* translation of truncated mRNA in a homologous cell-free translation system developed previously in our laboratory (11). Because the truncated RNA does not contain a stop codon, the nascent chain remains associated with the ribosome, and the signal peptide is exposed outside the ribosome that covers ~35 C-terminal amino acids. Translation was carried out in the presence of purified IMVs to allow targeting and membrane insertion. Subsequently, interactions of the nascent Hbp were fixed by using the membrane-permeable, lysine-specific cross-linking reagent DSS. Finally, the samples were extracted with sodium carbonate to separate membrane integrated from the soluble and peripheral membrane proteins.

TABLE I
Bacterial strains and plasmids used in this study

| Strain or plasmid	Relevant genotype	Reference
MC4100	F'*araD139* Δ(*argF-lac*)U169 *rpsL150 relAI flb5301 ptsF25 rbsR*	Ref. 26
TOP10F'	F'{*lacI*^{q} Tn*10*(Tc^R)} *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 deoR recAI*	Invitrogen
	araD139 Δ(*ara-leu*)7697 galU galK rpsL(Str^R) *endA1 nupG*	
BL21 (DE3)	F' *ompT hsdS*$_B$ (r$_B^-$ m$_B^-$) *gal dcm* Δ(*srl-recA*)306::Tn*10* (Tc^R) (DE3)	Ref. 27
HDB51	MC4100 *ara*$^+$ *secB*$^+$ *zic-4901*::Tn*10 ffh*::*kan-1* λ(P$_{ara}$-*ffh* Ap^r).	Ref. 28
HDB52	MC4100 *ara*$^+$ *secB*::Tn*5 zic-4901*::Tn*10 ffh*::*kan-1* λ(P$_{ara}$-*ffh* Ap^r)	Ref. 28
HDB97	MC4100 *malP*::*lacI* Δ*ara714 cps*::*lacZ mal*$^+$	Ref. 28
HDB107	MC4100 *malP*::*lacI* Δ*ara714 cps*::*lacZ mal*$^+$ Δ*lon510* Δ*clpYQ1172*::*tet*	Ref. 28
IQ85	*secY24*(ts) Tn*10 thiA* Δ*lac araD rspL rspE relA*	Ref. 29
IQ86	Tn*10 thiA* Δ*lac araD rspL rspE relA*	Ref. 29
MM152	MC4100 *zhe*::Tn*10 malT*^{C} *secB*::Tn*5*	Ref. 17
JARV16	MC4100 Δ*tatA* Δ*tatE*	Ref. 30
pET9-FtsY-A449	pET9, *ftsY-A449*	Ref. 18
pACYC-Hbp	pACYC184 (Ori$_{p15A}$), *hbp*	Ref. 31
pC4MethssHbp	pC4Meth, *ss-Hbp*	This study
pHB6.4-Hbp	pBR322 (Ori$_{ColE1}$), *hbp*	This study
pHB6.4-HbpΔss	pBR322 (Ori$_{ColE1}$), *hbp*Δ*ss*	This study
pHE12.6	pBR322 (Ori$_{ColE1}$), *hbp*	Ref. 1
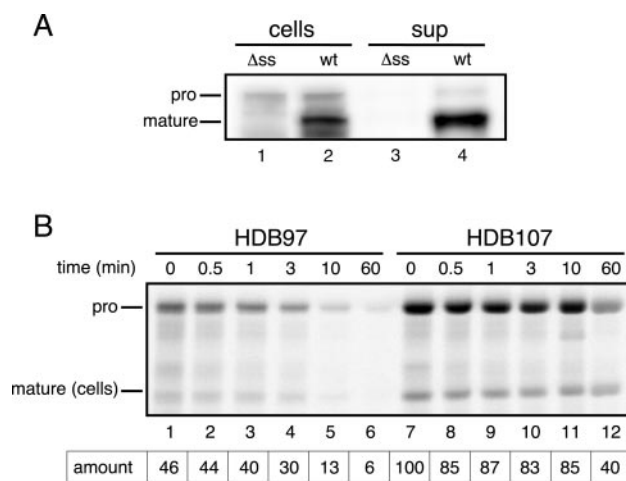
FIG. 2. *A,* the signal sequence of Hbp is required for secretion. Strain Top10F', harboring pHB6.4-Hbp (*wt,* wild-type) or pHB6.4-HbpΔss, was grown to an OD$_{660}$ of 0.5 and split in cells and supernatant (*sup*). Samples corresponding to 0.1 OD$_{660}$ unit were subjected to 8% SDS-PAGE and Western blotting using anti-Hbp serum. *B,* the proteases Lon and/or ClpYQ are involved in degradation of precursor Hbp in the cytosol. HDB97 (wild-type) and HDB107 (*lon*$^-$, *clpYQ*$^-$), harboring pHB6.4-HbpΔss, were grown to mid-log growth phase, radiolabeled with [^{35}S]methionine for 1 min, and chased for the times indicated. The cell samples were immunoprecipitated using anti-Hbp serum and subjected to SDS-PAGE. The numbers below the lanes show the relative quantified amount of pro-Hbp. *Lanes 1–6*, HDB97 cells; *lanes 7–12*, HDB107 cells. *pro*, pro-Hbp (142 kDa); *mature*, mature Hbp (111 kDa).

Approximately 30% of the synthesized nascent Hbp was detected in the carbonate pellet (Fig. 3, quantification data not shown). Given the relatively low intrinsic efficiency of the *E. coli in vitro* translocation system, this result indicated that nascent Hbp is properly targeted and inserted into the membrane and remains anchored via its signal peptide that is not cleaved at this nascent chain length. In the untargeted (carbonate soluble) fraction, a cross-linking product of ~60 kDa appeared that could be immunoprecipitated using serum directed against Ffh, the protein component of the SRP (Fig. 3, *lane 4*). The molecular mass of this product is consistent with the combined molecular mass of Ffh (50 kDa) and the Hbp 117-mer (13 kDa). The cross-linking to Ffh is remarkably strong considering the low abundance of the SRP in the translation lysate, suggesting that it represents a functional interaction. Weaker cross-linking products of slightly higher molecular mass were immunoprecipitated using anti-TF serum (Fig. 3, *lane 3*). TF (54 kDa) cross-linked to nascent chains often
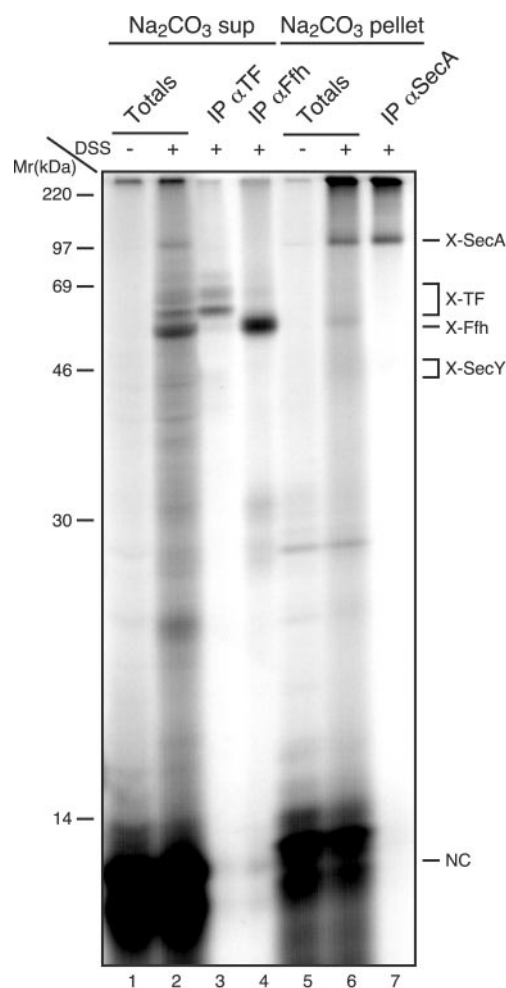

FIG. 3. **Nascent Hbp interacts with TF, SRP and SecA.** The 117-mer Hbp was synthesized in the presence of IMVs. After translation, samples were either treated with DSS (*lanes 2–4* and *6* and *7*) or mock treated (*lanes 1* and *5*) and subsequently extracted with carbonate (supernatant (*sup*), *lanes 1–4*; pellet, *lanes 5–7*). DSS-treated fractions were immunoprecipitated using antiserum against TF, Ffh, and SecA (*lanes 3, 4,* and *7*). The translation products at 30 kDa present in *lanes 5* and *6* represent the peptidyl-tRNA form of nascent Hbp. Cross-linked products and nascent chains (*NC*) are indicated.

migrates at varying positions (10). The membrane-integrated nascent Hbp was primarily cross-linked to SecA (102kDa) (Fig. 3, *lane 7*). In addition, a very faint cross-linking smear was
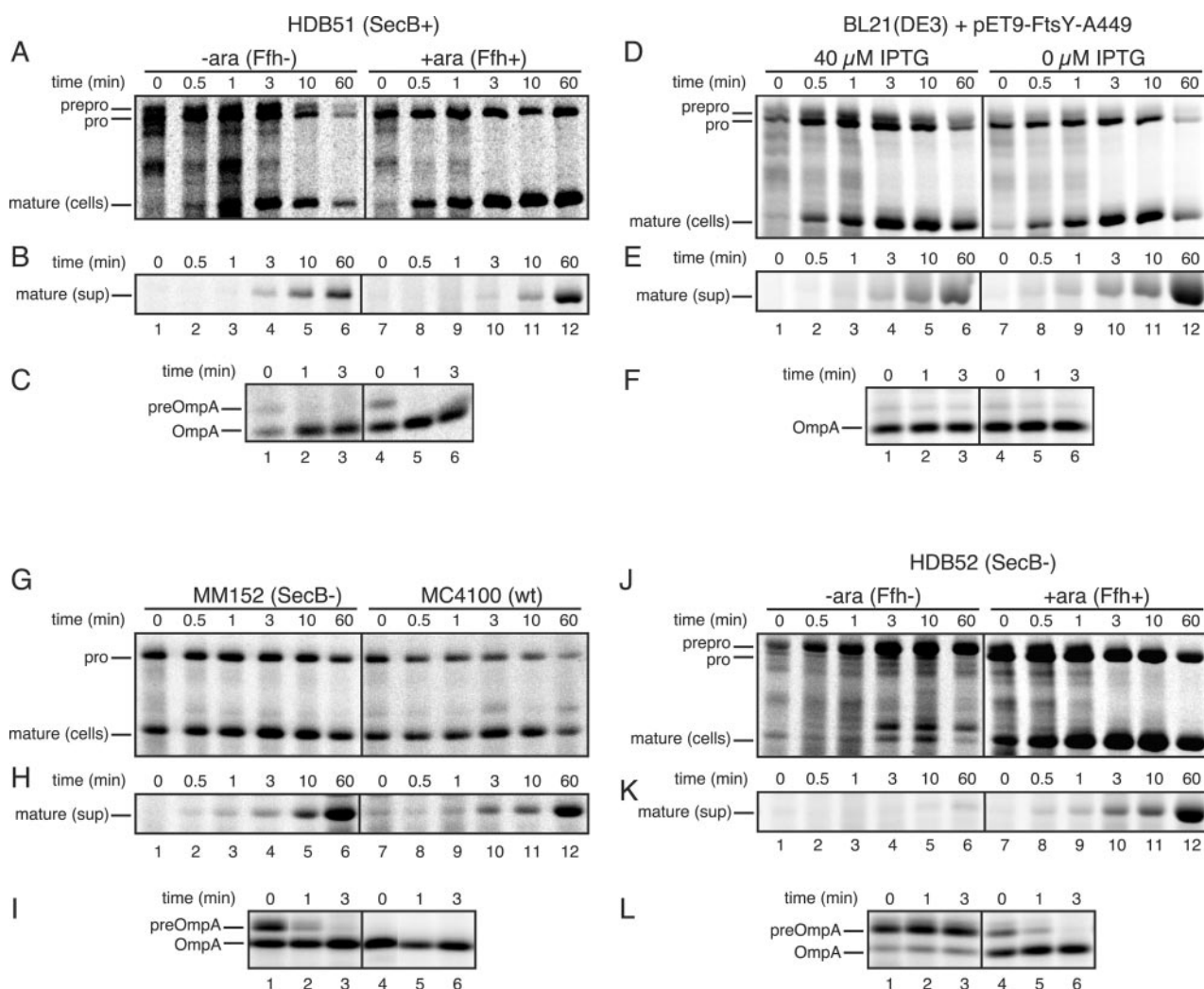
FIG. 4. **The SRP-pathway is required for efficient targeting of Hbp.** *A–C*, depletion of Ffh inhibits processing and secretion of Hbp. HDB51 (P$_{ara}$-*ffh secB$^+$*) harboring pACYC-Hbp was grown in M9 medium without (−*ara*) or with L-arabinose (+*ara*). Cells were radiolabeled with [^{35}S]methionine for 1 min and chased for the times indicated. *Panel A* shows Hbp immunoprecipitated from cell samples. *Panel B* shows trichloroacetic acid-precipitated proteins from the supernatant (*sup*), and *panel C* shows OmpA immunoprecipitated from cell samples. *D–F*, overexpression of mutant FtsY inhibits the processing and secretion of Hbp. BL21(DE3) harboring pET9-FtsY-A449 and pACYC-Hbp was grown in M9 medium. At an OD$_{660}$ of 0.2, the expression of FtsY-A449 was induced with 40 μM isopropyl-1-thio-β-D-galactopyranoside for 15 min or left uninduced as indicated. Samples were processed and displayed as described above. *G–I*, inactivation of SecB does not affect processing and secretion of Hbp. MM152 (*secB$^-$*), and MC4100 (*wt*) harboring pHE12.6 were grown in M9 medium. Samples were processed and displayed as described above. *J–L*, inactivation of SecB aggravates the effects of depletion of Ffh on the processing and secretion of Hbp. HDB52 (P$_{ara}$-*ffh secB$^-$*) harboring pACYC-Hbp was grown in M9 medium without (−*ara*) or with L-arabinose (+*ara*). Samples were processed and displayed as described above. *Prepro*, pre-pro-Hbp (148 kDa); *pro*, pro-Hbp (142 kDa); *mature*, mature Hbp (111 kDa).

observed at ~46 kDa that contained SecY, as evident from long exposures of immunoprecipitated samples (not shown).

Together, the cross-link patterns are reminiscent of those found with the nascent IMPs FtsQ and leader peptidase I (Lep) (10, 11, 14). Both FtsQ and leader peptidase I are targeted by the SRP to the Sec-translocon (15, 16). Apparently, nascent Hbp can be targeted to the inner membrane and inserts close to the Sec-translocon. The unprecedented strong cross-linking to Ffh, considering the extremely low abundance of Ffh in the translation lysate, suggests a high affinity of the Hbp signal peptide for the SRP and, consequently, a role for the SRP in the targeting of Hbp.

Hbp Requires the SRP for Optimal Processing and Secretion in Vivo—We investigated whether the interaction of nascent Hbp with the SRP observed *in vitro* reflects a dependence on the SRP targeting pathway for processing and secretion of the full-length protein *in vivo*. Strains that are conditional for the expression of targeting factors were used in pulse-chase experiments to analyze the effects on the kinetics of processing as

described above. Furthermore, spent medium of the pulse-chase samples was analyzed to monitor the secretion of mature Hbp in time.

Under normal conditions, N-terminal processing appeared to be very fast, and pre-pro-Hbp could only be detected in the pulsed sample (Fig. 4A, *lane 7*) as has been observed for many pre-secretory proteins such as OmpA (see also Fig. 4C, *lane 4*). However, C-terminal processing of Hbp is much slower. Under the expression conditions used, not all pro-Hbp was converted into mature Hbp even after 1 h of chase (Fig. 4A, *lane 12*). The actual release of mature Hbp into the culture medium is even slower, appearing prominently only after 1 h of chase (Fig. 4B, *lane 12*). Depletion of Ffh resulted in an accumulation of pre-pro-Hbp in the cells (Fig. 4A, *left panel*). The amount of secreted mature Hbp after 60 min of chase, but not the kinetics of the secretion of mature Hbp, appeared affected upon depletion of Ffh (Fig. 4B, *left panel*). As a control, the processing of OmpA was hardly influenced (Fig. 4C, *left panel*), which is in agreement with its requirement for SecB rather than SRP for tar-
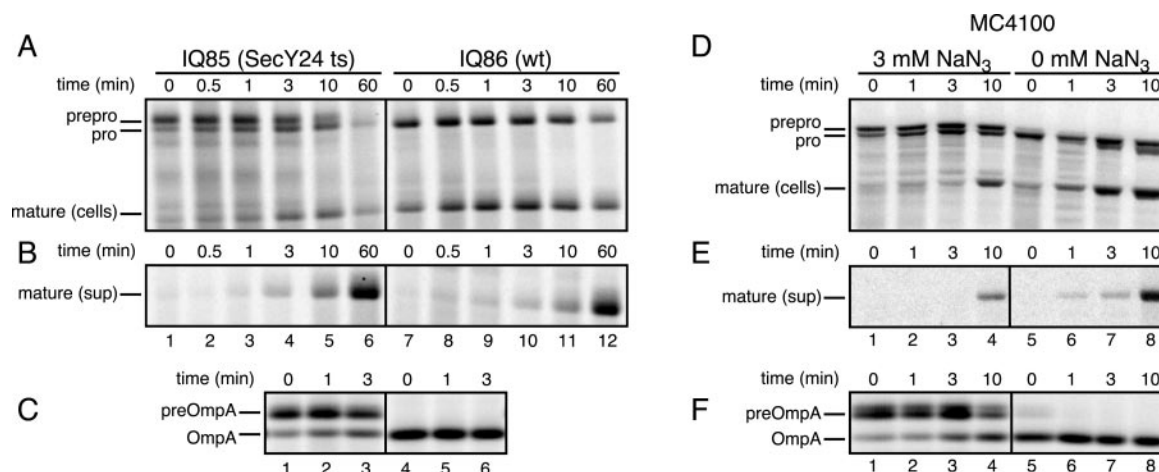
FIG. 5. **The Sec-translocon is required for efficient translocation of Hbp.** *A*, inactivation of SecY inhibits processing and secretion of Hbp. IQ85 (*secY24* ts) and IQ86 (*wt*, wild-type), harboring pHE12.6, were grown at 30 °C to an OD_{660} of 0.3 and shifted to 42 °C for 3 h. Samples were processed and displayed as described in the legend to Fig. 4. *B*, inhibition of SecA functioning with azide inhibits processing and secretion of Hbp. MC4100 (wild-type), harboring pHE12.6, was grown at 37 °C in M9 medium. Three minutes prior to labeling, 3 mM NaN_3 was added to inhibit the ATPase activity of SecA as indicated. Samples were processed and displayed as described in the legend to Fig. 4.

geting (17). In an alternative approach to analyzing the role of the SRP-targeting pathway in Hbp secretion, the effect of overexpression of FtsY-A449 was investigated. This mutant SRP receptor has a reduced GTP-binding capacity as a result of an amino acid substitution in the fourth GTP-binding consensus element (18). Moderate overexpression of FtsY-A449 has been shown to compromise SRP-mediated protein targeting (18). Hbp processing and, consequently, also secretion appeared to be impaired upon moderate overexpression of FtsY-A449 as opposed to the non-induced expression level (Fig. 4, *D* and *E*). OmpA processing was not affected under these conditions (Fig. 4*F*).

To examine whether the other major targeting factor, SecB, is also involved in Hbp targeting, Hbp was expressed in strain MM152, which lacks SecB, and in its isogenic wild-type strain, MC4100. Pre-pro-Hbp did not accumulate in the SecB minus strain, nor was the secretion of mature Hbp affected (Fig. 4, *G* and *H*). As a control, pre-OmpA accumulated at early chase times in strain MM152. Apparently, SecB is not necessary for efficient targeting of Hbp *per se*.

We next considered the possibility that the residual processing and secretion of Hbp under Ffh-deficient conditions is due to alternative targeting via SecB. Consistent with this explanation, the expression of Hbp in a double mutant strain (SecB knockout, Ffh conditional) showed a much stronger secretion phenotype upon Ffh depletion than the single (Ffh conditional) mutant (Fig. 4, *J* and *K*, *left panels*). The secretion defect was greatly reduced in cells that express Ffh (Fig. 4, *J* and *K*, *right panels*), again suggesting that SecB is not required for the targeting of Hbp under normal conditions. The accumulation of pre-pro Hbp under these conditions at early chase times remains unexplained but may be related to adverse effects of the unnatural control of Ffh expression from the arabinose promoter. Interestingly, a similar but opposite additive effect is observed for OmpA. When the preferred targeting factor, SecB, is absent, processing is impaired (Fig. 4*L*, *right panel*). Additional depletion of Ffh completely blocks residual OmpA processing (Fig. 4*L*, *left panel*).

Together, these results suggested that Hbp requires the SRP pathway for optimal targeting to the inner membrane. Although SecB is not essential for targeting *per se*, it can apparently compensate to a certain extent for depletion of the SRP.

Hbp Requires SecA and SecY for Efficient Processing and Secretion in Vivo—Two main types of translocons mediate the

transfer of proteins across the inner membrane, namely the Sec-translocon (6) and the Tat-translocon (19). We investigated the role of these translocons in the secretion of Hbp *in vivo* using the pulse-chase approach described above.

A temperature-dependent conditional *secY* mutant strain was used to deplete the cells for functional Sec-translocons. At the non-permissive temperature, processing of the control Sec substrate OmpA was severely impaired in this strain as compared with its parental wild-type strain (Fig. 5*C*). Likewise, pre-pro-Hbp accumulated in the *secY* Ts cells (Fig. 5*A*, *left panel*), indicating that translocation of Hbp across the inner membrane proceeds through the Sec-translocon.

To study the role of SecA, its ATPase activity was perturbed with azide. Under the conditions used, processing of OmpA was almost completely inhibited (Fig. 5*F*, *left panel*). Again, pre-pro-Hbp accumulated in the cells, indicating that translocation of Hbp is dependent on SecA (Fig. 5*A*, *left panel*). A similar dependence on functional SecA was observed when a temperature-dependent conditional *secA* mutant strain was used (data not shown).

The Tat-translocon is used by a subset of preproteins that are folded prior to translocation (19). Tat substrates carry an essential twin arginine motif in their signal peptide just upstream of the hydrophobic domain. Although the Hbp signal peptide does not fully comply with this motif, it does contain two consecutive arginine residues upstream of the hydrophobic core region. This feature prompted us to investigate a possible role of the Tat-translocon using a strain that lacks TatA and TatE, rendering it completely unable to translocate Tat substrates. This double mutant strain showed normal kinetics of processing and secretion of Hbp whereas processing of the known Tat substrate SufI was completely blocked, arguing that the Tat-translocon is not involved in secretion of Hbp (data not shown).

Together, the data suggest that Hbp uses the Sec-translocon for transfer across the inner membrane consistent with the *in vitro* cross-link data (Fig. 3). SecA appears to be required to drive the translocation process.

DISCUSSION

In the present work, we have addressed the question how the autotransporter Hbp is targeted to and translocated across the inner membrane. Both *in vitro* cross-linking and *in vivo* pulse-chase labeling experiments point to the use of a co-transla-

tional targeting and translocation mechanism involving the targeting factor SRP and the Sec translocation machinery. This is the first example of an extracellular protein that can be targeted by the SRP. Interestingly, in the absence of a functional SRP pathway, part of the mistargeted Hbp is rescued by SecB, underscoring the inherent flexibility of protein targeting in *E. coli* (20).

What are the features in the Hbp signal peptide that determine SRP binding? Previous work in our group has demonstrated that the SRP preferentially interacts with relatively hydrophobic signal peptides such as those that are present in IMPs (10). However, the hydrophobic core region at the C terminus of the Hbp signal peptide is not particularly hydrophobic. Interestingly, the Hbp signal peptide is relatively long (52 amino acids) and appears to contain an N-terminal extension that precedes a "classical" signal peptide (Fig. 1). It is attractive to speculate that the N-terminal extension plays a role in the recognition by the SRP either directly or indirectly by presenting the hydrophobic core in a favorable conformation or by recruiting other factors that increase the affinity of the Hbp signal peptide for the SRP. It is worth mentioning that the only other known example of a secreted protein that makes use of the SRP for targeting, SecM, is also synthesized with a long signal peptide that comprises an N-terminal extension and a moderately hydrophobic core region (21). SecM is a regulatory protein that functions in the secretion-responsive control of SecA expression. In wild-type cells, SecM is translocated to the periplasm where it is rapidly degraded (22).

Alternatively, the N-region (KCVHKSVRR) between the hydrophobic core and the N-terminal extension might be important for SRP recognition of the Hbp signal peptide. Compared with other signal peptides in Gram-negative bacteria, this region is more basic. Interestingly, the crystal structure of the SRP has revealed an unusual RNA-protein interface that is thought to constitute the signal peptide binding groove (23). It has been suggested that the protein moiety of the interface interacts with the hydrophobic core of the signal peptide, whereas the RNA is responsible for recognizing the basic N-domain. Following this reasoning, a more basic N-domain might compensate for a less hydrophobic core region in SRP binding. These possibilities are currently being investigated.

Translocation of autotransporters across the inner membrane has been proposed to involve the N-terminal signal peptide and occur via the Sec-pathway, which is also used by periplasmic and outer membrane proteins (3). Consistent with this proposal, our data suggest that the Sec-translocon receives and translocates the nascent Hbp. In this respect, Hbp resembles IMPs like leader peptidase I, FtsQ, and MtlA (7). An accessory translocon component, YidC, is specifically involved in membrane integration of these IMPs but not in the translocation of secretory proteins (11, 24).[2] We have not observed any cross-linking of nascent Hbp with YidC. Moreover, depletion of YidC did not affect processing and secretion of Hbp (data not shown). Apparently, YidC is dispensable for the reception of Hbp at, as well as the translocation of Hbp across, the inner membrane-embedded Sec-translocon.

It is not unlikely that other members of the autotransporter family follow the same pathway of targeting and translocation across the bacterial inner membrane. Many autotransporters carry signal peptides of similar length and domain structure (3). The N-terminal extension in these signal peptides is remarkably conserved, as is the basic character of the N-domain.

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[2] E. N. G. Houben, personal communication.

In addition, substrates of an analogous secretion system, the "two-partner secretion" (TPS) pathway in which the β-barrel domain is present in a separate protein, also possess signal peptides that are conserved with members of the classical autotransporter family (5). One of these substrates, the HMW1 adhesin from *Haemophilus influenzae* that carries a 68 amino acid-long signal peptide was shown to require SecA and SecE for maturation and secretion (25).

What would be the benefit of co-translational translocation for autotransporters? For Hbp, it might prevent degradation or premature folding of Hbp in the cytoplasm. Hbp that lacks its signal peptide appeared to be vulnerable to degradation by cytoplasmic proteases. It should be noted that both the autotransporter and two-partner secretion families comprise many virulence factors such as hemagglutinins, hemolysins, cytolysins, and proteases (5) that may be harmful when expressed in the cytoplasm of the pathogenic bacterium. Furthermore, the autotransporters are relatively large molecules with a typical domain structure. The passenger domain that is expressed in the cytoplasm may fold into a conformation that is incompatible with translocation through the Sec-translocon, even when the β-barrel domain is still being synthesized.

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