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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.

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 secreted by a human pathogenic Escherichia coli strain (1) and contributes to the pathogenic synergy between E. coli and Bacteroides fragilis in intra-abdominal infections (2). It represents the first described member of the serine protease autotransporters of Enterobacteriaceae (SPATE) group of autotransporter proteins (3).

The N-terminal domain is thought to function as a signal peptide to direct 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). The periplasmic 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 (6).

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 Buf 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 a C/4Met-Hbp, 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
possessing extended signal peptides is given together with a consensus comparison of the N-terminal extensions of several autotransporters.

...lacking L-arabinose, and diluted to an OD660 of 0.004 in M9 min. To inhibit SecA functioning in MC4100, 3 mM NaN3 was added 3 min prior to labeling. In all experiments, 2.4 OD660 units of cells were added to induce FtsY-A449 expression, and growth was continued for 15 min...
**Targeting and Translocation of the Autotransporter Hbp**

**TABLE I**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC4100</td>
<td>F' araD39 ∆argE-lacU169 rpsL150 relAI fba501 ptsP25 rbsR</td>
<td>Ref. 26</td>
</tr>
</tbody>
</table>
| TOP10F             | F' [ lacI1 (Tn10(Trc3)) ] mcrA ∆mrr-hsdRMS-mcrBC ΔF800lacZΔM15 ΔlacX7 deoR recA1 araD139 Δara-leu7687 galU galK rpsL5050 strept 
_sed110 supE44 endA1 supF uvrB4 leuB6 thi-1 hsdS29 thy-1 (strR' lacI1 
 lacZΔM15 galK8 rpsL5050 strA1575) ] | Invitrogen |
| BL21 (DE3)        | F' ompT hsdS(rC, mIV) gal dcm Δ(rpsL)A396::Tn10 (Ts) (DE3) | Ref. 27 |
| HDB51             | MC4100 ara- secB+ zic-4901::Tn10 [fha-kan-1 λP_[fha]] Apr' | Ref. 28 |
| HDB52             | MC4100 ara- secB+ Tet:5 [fha-kan-1 λP_[fha]] Apr' | Ref. 28 |
| HDB97             | MC4100 malP::lacΔara714 eps::lacZ mal:: λlon510 ΔclpYQ1172::tet | Ref. 28 |
| HDB107            | MC4100 malP::lacΔara714 eps::lacZ mal:: λlon510 ΔclpYQ1172::tet | Ref. 28 |
| IQ85              | secY2A(ts) Tn10 thia Δlac araD rpsL rpsE relA | Ref. 29 |
| IQ86              | Tn10 thia Δlac araD rpsL rpsE relA | Ref. 29 |
| MM152             | MC4100 zhc::Tn10 malI+ secE::Tn5 | Ref. 17 |
| JARV16            | MC4100 ΔaraA ΔmalE | Ref. 30 |
| pC4MethsHbp       | pC4Meth, ss-Hbp | Ref. 18 |
| pACYC-Hbp         | pACYC184 (OrI327A), hbp | Ref. 31 |
| pC4MethsHbp       | pC4Meth, ss-Hbp | This study |
| pH64-Hbp          | pBR322 (OrI321), hbp | This study |
| pH64-HbpΔss       | pBR322 (OrI321), hbpΔss | This study |
| pH12.6            | pBR322 (OrI321) | 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 OD₆₆₀ of 0.5 and split in cells and supernatant (sup). Samples corresponding to 0.1 OD₆₆₀ unit were subjected to 6% 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 pH64.4-HbpΔss, were grown to mid-log growth phase, radiolabeled with [³⁵S]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 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...
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 pre- and post-translocational events. 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 (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-
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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 function 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 NaN₃ was added to inhibit the ATPase activity of SecA as indicated. Samples were processed and displayed as described in the legend to Fig. 4.

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, A and E). Moderate overexpression of FtsY-A449 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. 5C). Likewise, pre-pro-Hbp accumulated in the secY Ts cells (Fig. 5A, left panel), indicating that translocation of Hbp across the inner membrane proceeds through the Sec-translocon.

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. 4L, right panel). Additional depletion of Ffh completely blocks residual OmpA processing (Fig. 4L, 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.

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-
The 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 IMFs (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 (KCVHKVSRR) 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, PtsQ, and MlaA (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). 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.

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, cytolsins, 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.

REFERENCES


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