# Targeting and Translocation of Two Lipoproteins in *Escherichia coli* via the SRP/Sec/YidC Pathway*

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In *Escherichia coli,* two main protein targeting pathways to the inner membrane exist: the SecB pathway for the essentially posttranslational targeting of secretory proteins and the SRP pathway for cotranslational targeting of inner membrane proteins (IMPs). At the inner membrane both pathways converge at the Sec translocase, which is capable of both linear transport into the periplasm and lateral transport into the lipid bilayer. The Sec-associated YidC appears to assist the lateral transport of IMPs from the Sec translocase into the lipid bilayer. It should be noted that targeting and translocation of only a handful of secretory proteins and IMPs have been studied. These model proteins do not include lipoproteins. Here, we have studied the targeting and translocation of two secretory lipoproteins, the murein lipoprotein and the bacteriocin release protein, using a combined *in vivo* and *in vitro* approach. The data indicate that both murein lipoprotein and bacteriocin release protein require the SRP pathway for efficient targeting to the Sec translocase. Furthermore, we show that YidC plays an important role in the targeting/translocation of both lipoproteins.

In the bacterium *Escherichia coli*, the SecB pathway targets a subset of secretory proteins to the Sec translocase (1). The chaperone SecB keeps secretory proteins in a translocation-competent state. The SecB-preprotein complex is targeted at a late stage during translation or after translation to the Sec translocase in the inner membrane. The signal recognition particle (SRP)[1] pathway targets IMPs to the same or a very similar Sec translocase in a cotranslational mechanism (2, 3). The SRP, which consists of the Ffh protein and the 4.5 S RNA, binds to the first transmembrane segment (TM) of an IMP when it becomes exposed outside the ribosome. Upon contact of the SRP with its receptor, FtsY, the nascent IMP dissociates from the SRP and enters the Sec translocase.

The core of the Sec translocase consists of the IMPs, SecY and SecE, and the peripheral subunit, SecA (1). SecY and SecE form a protein-conducting channel (4), and SecA drives proteins in an ATP-dependent process through this channel (1). The Sec translocase catalyzes linear transport of secretory proteins and of periplasmic domains of IMPs across the membrane. In addition, TMs of IMPs are recognized in the Sec translocase and laterally transferred into the lipid bilayer. The Sec translocase-associated form of YidC appears to assist in this lateral transfer of TMs (5, 6). YidC, which is present in excess over the Sec translocase, is also involved in the integration of some small SRP/Sec-independent IMPs (7, 8). Thus far, no evidence has been obtained that points to a role of YidC in the targeting/translocation of secretory proteins across the inner membrane (7, 9).[2]

It should be noted that in *E. coli* targeting and translocation of only a handful of secretory proteins and IMPs have been studied thoroughly. Hardly anything is known about the targeting and translocation of lipoproteins, which in most cases are secretory proteins. A lipoprotein is synthesized as a preprotein with an N-terminal signal sequence. Lipoproteins contain a conserved sequence, the "lipobox," that includes the signal peptidase II (SPase II) cleavage site (10). The cysteine located just after the SPaseII cleavage site is diacylglycerated upon translocation; subsequently the signal sequence is clipped off by SPaseII, yielding an apolipoprotein. Finally, the amino-modified cysteine is fatty acylated, giving rise to the mature lipoprotein (11). Secretory lipoproteins can, depending on the sequence of the early mature region, remain associated to the outer leaflet of the inner membrane or be transported by the Lol system to the outer membrane (12).

To identify the components involved in the targeting and translocation of secretory lipoproteins, we have analyzed the maturation of two model secretory lipoproteins. Maturation of lipoproteins has been studied *in vivo* using strains that are mutated in targeting and translocation factors and *in vitro* using a translation/cross-linking system. One of the two model lipoproteins is the murein lipoprotein (Lpp), which is the most abundant protein in *E. coli*. Lpp is attached to the inner leaflet of the outer membrane of *E. coli* and forms stable trimers (13). One of three Lpp molecules is covalently linked to the peptidoglycan layer (13). The other model lipoprotein is the pCloCF13-encoded bacteriocin release protein (BRP) (14). The BRP is essential for the translocation of the bacteriocin cloacin

[1] The abbreviations used are: SRP, signal recognition particle; IMP, inner membrane protein; IMV, inverted membrane vesicle; IPTG, isopropyl-1-thio-β-D-galactopyranoside; LP, mature lipoprotein; OmpA, outer membrane protein A; TF, trigger factor; TM, transmembrane segment; (Tmd)Phe, L-[3-(trifluoromethyl)-3-diazirin-3H-yl]phenylalanine; U-PLP, unmodified prolipoprotein; HA, hemagglutinin; BRP, bacteriocin release protein; Lpp, murein lipoprotein; SPase, signal peptidase.

[2] E. N. G. Houben and J. Luirink, unpublished observations.

DF13, a bactericidal protein, across the cell envelope (14). Notably, the BRP signal sequence is very stable after cleavage from the preprotein, in contrast to other signal sequences, and plays a yet undefined role in cloacin DF13 export (14).

Our combined *in vivo* and *in vitro* studies indicate that the SRP pathway plays an important role in the targeting of Lpp and BRP to the Sec translocase. Surprisingly, YidC is also shown to function in the targeting/translocation of both secretory lipoproteins.

<center>MATERIALS AND METHODS</center>

*Reagents, Enzymes, and Sera*—All restriction enzymes, T4 DNA ligase, and alkaline phosphatase were purchased from Invitrogen. The Expand long template PCR kit was from Roche Applied Science. The QuikChange site-directed mutagenesis kit was from Stratagene, and the Megashort script T7 transcription kit was from Ambion Inc. [35S]methionine and protein A-Sepharose were from Amersham Biosciences. Pansorbin was obtained from Merck. All other chemicals were supplied by Sigma. Antiserum against hemagglutinin (HA) tag was purchased from Sigma and AbCam. Antisera against L23 and L29 were kind gifts from R. Brimacombe. The antisera against TF and SecA were gifts from W. Wickner. Antisera against Ffh, YidC, and SecY were from our own collection.

*E. coli Strains, Plasmids, and Growth Conditions for in Vivo Targeting and Translocation Studies*—*E. coli* strain TOP10F′ grown in Luria Bertani medium was used for all plasmid constructions. In all lipoprotein expression vectors, the pCloDF13-encoded T1 terminator, which regulates the expression of the pCloDF13-encoded BRP, was cloned upstream of the genes encoding Lpp and BRP to prevent any background expression (15). Upon induction of expression, the T1 terminator is "overruled." The pCloDF13-encoded T1 terminator region was amplified using pJL28 as a template (16). Primers were designed so that both an NcoI site and a stop codon were introduced upstream and an EcoRI site and a ribosome binding site were introduced downstream of the T1 terminator region. The T1 terminator region was cloned NcoI-EcoRI into pET21d, yielding pET21d-T1. Plasmids pET21d-T1Lpp and pET21d-T1BRP were obtained by plasmid PCR and site-directed mutagenesis using pGEM42-Lpp and pJL28, respectively, as templates (17). A methionine codon was introduced at position 16 in the Lpp signal sequence and position 17 in the BRP signal sequence (Fig. 1). In addition, a C-terminal 4 × methionine tag was attached to both Lpp and BRP to increase labeling efficiency. Subsequently, T1Lpp and T1BRP were NcoI-HindIII-cloned into pEH1 (18), pEH3 (18), and pBAD24 (19), yielding pEH1-T1Lpp, pEH3-T1Lpp, pBAD24-T1Lpp, pEH1-T1BRP, pEH3-T1BRP, and pBAD24-T1BRP. Finally, the genetic information coding for an HA tag with a stop codon at its 3′ prime end (20), preceded by a flexible linker (Pro-Gly-Gly) was fused to the 4 × methionine-tagged Lpp and BRP, using BamHI and HindIII sites. This yielded pEH1-T1LppHA, pEH3-T1LppHA, pBAD24-T1LppHA, pEH1-T1BRPHA, pEH3-T1BRPHA, and pBAD24-T1BRPHA. The nucleotide sequences of all constructs were verified by DNA sequencing.

The Ffh conditional strain WAM121 was cultured in M9 minimal medium supplemented with 0.2% arabinose as described previously (21). To deplete cells for Ffh, cells were grown to mid-log phase in the absence of arabinose. The 4.5 S RNA conditional strain FF283 was cultured in M9 minimal medium supplemented with 1 mM IPTG as described previously (21). To deplete cells for 4.5 S RNA, cells were grown to mid-log phase in the absence of IPTG. The temperature-sensitive amber suppressor SecA depletion strain BA13 and the control strain DO251 were cultured in M9 minimal medium at 30 °C as described previously (22, 23). To deplete cells for SecA, cells were grown to mid-log phase at 41 °C. The SecE depletion strain CM124 was cultured in M9 minimal medium supplemented with 0.2% glucose and 0.2% L-arabinose as described previously (24, 25). To deplete cells for SecE, cells were grown to mid-log phase in the absence of L-arabinose. The temperature-sensitive amber suppressor YidC depletion strain KO1672, along with its wild-type control strain KO1670 (26), were cultured in M9 minimal medium at 30 °C overnight. To deplete KO1672 cells for YidC, cells were grown to mid-log phase at 42 °C. Where appropriate, ampicillin (100 $\mu$g ml$^{-1}$), chloramphenicol (30 $\mu$g ml$^{-1}$), kanamycin (50 $\mu$g ml$^{-1}$), and tetracyclin (12.5 $\mu$g ml$^{-1}$) were added to the medium.

*In Vivo Assay for Targeting and Translocation*—The model lipoproteins Lpp and BRP were expressed by L-arabinose induction from the pBAD24 vector in strains TOP10F′, FF283, BA13, DO251, KO1672, and KO1670 and by IPTG induction from the pEH1 vector in strain CM124

and the pEH3 vector in strain WAM121. For all experiments cells were grown to mid-log phase. Expression of the constructs was induced for 3 min with either L-arabinose (0.2%) or IPTG (1 mM). When indicated, the SPaseII inhibitor globomycin (final concentration 100 $\mu$g ml$^{-1}$) was added 5 min before induction. Cells were labeled with [35S]methionine (60 $\mu$Ci/ml, Ci = 37 GBq) for 30 s before precipitation with trichloroacetic acid (final concentration 10%). Subsequently, the samples were washed with acetone, resuspended in 10 mM Tris/2% SDS, and immunoprecipitated with anti-HA and anti-OmpA serum. Anti-HA immunoprecipitates were analyzed by means of Tricine SDS-PAGE (16.5% peptide criterion gels from Bio-Rad) and anti-OmpA immunoprecipitations by means of standard SDS-PAGE. Gels were scanned by Fuji FLA-3000 phosphorimaging using the Image Reader V1.8J/Image Gauge V 3.45 software.

*E. coli Strains, Plasmids, and Growth Conditions for in Vitro Studies*—*E. coli* strain MC1061 grown in Luria Bertani medium supplemented with CaCl$_2$ (10 mM) was used for all plasmid constructions. The QuikChange site-directed mutagenesis kit was used for the construction of all point mutations. Strain MRE600 was used to prepare a lysate for translation of *in vitro* synthesized mRNA and suppression of UAG stop codons in the presence of (Tmd)Phe-tRNA$^{sup}$. Inverted membrane vesicles (IMVs) were prepared from strain MC4100 grown in Luria Bertani medium.

Plasmid pC4Meth55LppTAG11 (Fig. 1) was constructed by plasmid PCR and site-directed mutagenesis using pGEM42-Lpp as template (17). Plasmid pC4Meth55BRPTAG10 was obtained by plasmid PCR and site-directed mutagenesis using pJL28 as a template (16). pC4Meth55LppTAG11 encodes truncated Lpp, and pC4Meth55BRPTAG10 encodes truncated BRP. A methionine has been introduced at position 16 in the Lpp signal sequence and at position 17 in the BRP signal sequence, and both Lpp and BRP have been fused to a C-terminal 4 × methionine tag to improve labeling efficiency. Plasmid pC4Meth55LppTAG11 contains an amber mutation at position 11 in the Lpp gene, and plasmid pC4Meth55BRPTAG10 contains an amber mutation (TAG) at position 10 in the BRP gene to enable tRNA$^{sup}$ photocross-linking (Fig. 1). Where appropriate, ampicillin (100 $\mu$g ml$^{-1}$) was added to the medium.

*In Vitro Transcription, Translation, Targeting, and Cross-linking*—Truncated mRNA was prepared as described previously from HindIII-linearized Lpp and BRP derivative plasmids. For photocross-linking, (Tmd)Phe was site-specifically incorporated into nascent chains by suppression of a UAG stop codon using (Tmd)Phe-tRNA$^{sup}$ in an *E. coli in vitro* translation system containing [35S]methionine to label the nascent chains. This procedure has been described previously (5, 27). Targeting to IMVs, photocross-linking, and carbonate extraction (to separate soluble and peripheral membrane proteins from integral membrane proteins) was carried out as described previously (27). Carbonate-soluble and -insoluble fractions were either trichloroacetic acid precipitated or immunoprecipitated. The material used for immunoprecipitation was 2-fold the amount used for trichloroacetic acid precipitation. Release of nascent chains from the ribosome was provoked by incubating the nascent chains after translation for 10 min at 37 °C with EDTA (25 mM). Samples were analyzed using 15% Laemmli SDS-PAGE and phosphorimaging as described previously (27).

<center>RESULTS</center>

*Model Lipoproteins*—We have used the murein Lpp and the pCloDF13-encoded BRP as model proteins to study the targeting and translocation of secretory lipoproteins in *E. coli* (13, 14). To improve the labeling of Lpp and BRP with [35S]methionine, both lipoproteins were slightly modified. A methionine was introduced in the signal sequences of both Lpp and BRP (Fig. 1). This does not have a significant impact on the predicted hydrophobicity of the Lpp and BRP signal sequences, and we felt confident that the introduction of a methionine would affect Lpp and BRP signal sequence interactions only marginally at the most. Actually, in the ColA and ColN BRPs, which are homologous to the pCloDF13 BRP, a methionine naturally occurs at this position. To further improve labeling, a stretch of 4 methionines was attached to the very C terminus of both Lpp and BRP. To be able to immunoprecipitate Lpp and BRP in the *in vivo* experiments, the 9-amino acid-long influenza virus HA epitope tag preceded by a flexible linker (Pro-Gly-Gly) was attached to their C termini (Fig. 1) (20).

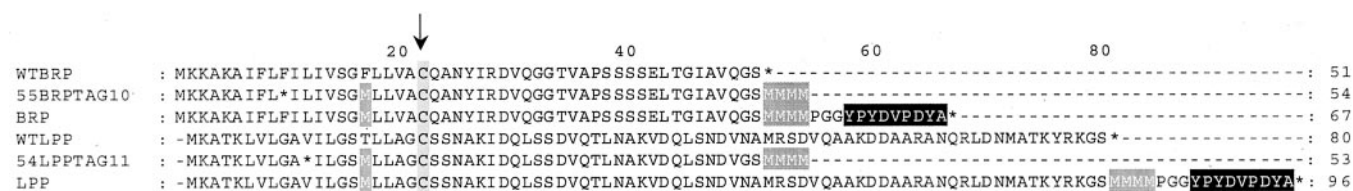The modified pCloDF13-encoded BRP causes lysis upon over-

FIG. 1. **The model lipoproteins Lpp and BRP.** The amino acid sequence of BRP, Lpp, and their derivatives used in this study: wild-type BRP (*WTBRP*), *in vitro* construct 55BRPTAG10, *in vivo* construct BRP, wild-type Lpp (*WTLPP*), *in vitro* construct 54LPPTAG11, *in vivo* construct Lpp (*LPP*). The SPaseII cleavage site is indicated by an *arrow*, the lipid modifiable cystein by *C*, inserted/added methionines by *M*, and attached influenza virus hemagglutinin epitope tags with a *black* background. Between the methionine stretch and the HA tag there is a flexible linker (*PGG*). Amber codons and stop codons are marked with an *asterisk*.

expression, just like the wild-type version of the protein, and the clipped off signal sequence is stable (results not shown). For the sake of clarity, we refer in the rest of this report to the modified lipoproteins as Lpp and BRP.

*Translocation of Lpp and BRP across the Inner Membrane Is Sec Translocase-dependent*—To test whether the introduction of the extra methionines and the HA tag interfere with the *in vivo* processing and maturation of Lpp and BRP, the constructs were studied in the *E. coli* TOP10F′ strain and Sec translocase mutant strains. Maturation of lipoproteins occurs in three steps: 1) the unmodified prolipoprotein (U-PLP) is converted into a diacylated prolipoprotein (M-PLP) upon translocation across the inner membrane, 2) cleavage of the signal sequence by SPaseII yields the apolipoprotein, and 3) acylation of the lipoprotein gives rise to the mature lipoprotein (11). Both Lpp and BRP were expressed in the TOP10F′ strain in the absence and presence of globomycin, which inhibits SPaseII. Inhibition of SPaseII causes the accumulation of the M-PLP form of lipoproteins (Fig. 2, *lanes 1* and *2*) (28). This was confirmed in experiments where [$^3$H]palmitate was used rather than [$^{35}$S]methionine to label cells in the presence of globomycin (results not shown). Taken together, the modifications improve labeling and facilitate immunoprecipitation of Lpp and BRP without affecting their maturation.

Lpp and BRP were also expressed in SecE and SecA depletion strains. Both SecE and SecA are key components of the Sec translocase. Upon depletion of SecE, SecY is rapidly degraded by the FtsH protease. Therefore, SecE depletion results in the loss of the SecY/E core of the Sec translocase (29). SecA depletion results in the loss of the motor of the Sec translocase (22). Upon SecE and SecA depletion, the U-PLP form of both Lpp and BRP accumulate (Fig. 2, *lanes 3–6*). This points to a key role of the Sec translocase in the translocation of Lpp and BRP across the inner membrane, corroborating previous studies using Sec-conditional mutant strains (30–33).

*Efficient in Vivo Targeting of Lpp and BRP Requires SRP*— How are Lpp and BRP targeted to the Sec translocase? The SecB and the SRP pathways are the two main targeting pathways to the Sec translocase (34). In the absence of SecB, targeting of both Lpp and BRP is not significantly affected (results not shown).

We next investigated the role of the SRP in the targeting of Lpp and BRP to the Sec translocase. The *E. coli* SRP consists of the protein component Ffh and the RNA component 4.5 S RNA. Both Ffh and 4.5 S RNA are essential for viability, and depletion of either of the SRP components compromises the SRP targeting pathway, thereby preventing the targeting of many IMPs (3, 35, 36). Targeting of Lpp and BRP was studied both under 4.5 S RNA and Ffh depletion conditions (Fig. 3, *A* and *B*). Depletion of 4.5 S RNA (Fig. 3*A*) and of Ffh (Fig. 3*B*) both resulted in accumulation of the unmodified precursor forms of BRP (most pronounced) and Lpp (less pronounced). As a control, the processing of pro-OmpA, an outer membrane protein that is targeted by SecB, was monitored in the same samples.
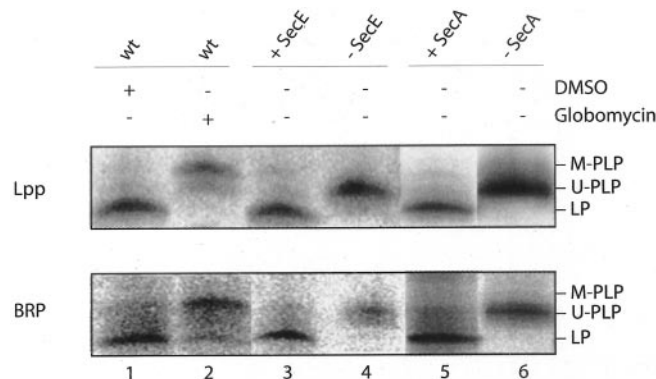


FIG. 2. **Translocation of Lpp and BRP is affected by the SPaseII inhibitor globomycin and is Sec translocase-dependent.** TOP10F′ cells harboring either pBAD24-T1LppHA (*top panel*, *lanes 1* and *2*) or pBAD24-T1BRPHA (*bottom panel*, *lanes 1* and *2*) were cultured in M9 medium and pulse-labeled in the absence and presence of the SPaseII inhibitor, globomycin. CM124 (P$_{Ara}$-*secE*) cells harboring either pEH1-T1LppHA (*top panel*, *lanes 3* and *4*) or pEH1-T1BRPHA (*bottom panel*, *lanes 3* and *4*) were cultured in M9 medium with (+*SecE*) or without (−*SecE*) L-arabinose. BA13 (SecA depletion strain, −*SecA*) cells and DO251 (control strain, +*SecA*) cells harboring either pBAD24-T1LppHA (*top panel*, *lanes 5* and *6*) or pBAD24-T1BRPHA (*bottom panel*, *lanes 5* and *6*) were cultured in M9 medium at 41 °C (SecA depletion conditions, −*SecA*). Cells were pulse-labeled and processed as described under "Materials and Methods." Lpp and BRP were immunoprecipitated with anti-HA antiserum. The processing of Lpp and BRP was not affected by Me$_2$SO (*DMSO*) in which globomycin was dissolved. The processing of the SpaseII-independent OmpA was not affected in the presence of globomycin, and depletion of SecE and SecA was checked by monitoring the accumulation of OmpA (results not shown).

No effect of depletion of the SRP components could be detected, confirming that the observed accumulation of U-PLP is not because of more general secondary effects of SRP depletion. Together, the results indicate that a functional SRP pathway is required for efficient targeting of the BRP and, albeit to a lesser extent, of the Lpp.

*Efficient in Vivo Translocation of Lpp and BRP Requires YidC*—All IMPs studied so far require YidC for efficient assembly into the inner membrane. It has been suggested that YidC assists the transfer of TMs from the Sec translocase into the lipid bilayer (5). So far, no evidence has been obtained pointing to a role of YidC in the translocation of secretory proteins (6, 7, 27, 37, 38). However, the unexpected role of the SRP in the targeting of BRP and Lpp prompted us to evaluate the role of YidC in the translocation of these proteins using a temperature-sensitive strain that is conditional for YidC expression (Fig. 4). To our surprise, depletion of YidC by growth at the non-permissive temperature resulted in the accumulation of unmodified precursor forms of BRP (most pronounced) and Lpp (less pronounced). Again, the processing of pro-OmpA that is translocated independent of YidC (7) was monitored as a control and appeared unaffected. The combined data suggest that YidC plays a differential role in the translocation of both lipoproteins.
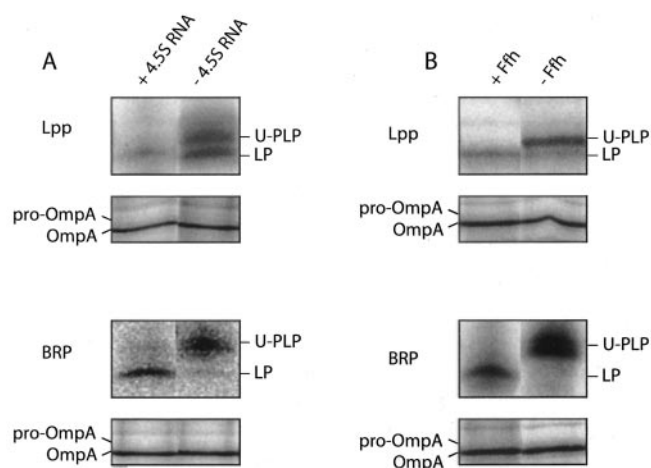
FIG. 3. **The SRP pathway is required for efficient targeting of Lpp and BRP.** *A*, FF283 (P$_{IPTG}$-*4.5 S RNA*) cells were cultured in M9 medium with (+*4.5 S RNA*) or without (−*4.5 S RNA*) IPTG. Cells were pulse-labeled and processed as described under "Materials and Methods." Lpp and BRP were immunoprecipitated with anti-HA antiserum and OmpA with OmpA antiserum. OmpA processing was not affected upon 4.5 S RNA depletion. *B*, WAM121 (P$_{ara}$-*ffh*) cells harboring either pEH3T1LppHA or pEH3T1BRPHA were cultured in M9 medium with (+*Ffh*) or without (−*Ffh*) arabinose. Cells were pulse-labeled and processed as described under "Materials and Methods." Lpp and BRP were immunoprecipitated with anti-HA antiserum and OmpA with OmpA antiserum. OmpA processing was not affected upon Ffh depletion.



FIG. 4. **YidC is required for efficient targeting of Lpp and BRP.** KO1670 (control strain, +*YidC*) cells and KO1672 (YidC depletion strain, −*YidC*) cells were cultured in M9 medium at 42 °C (YidC depletion conditions) and 30 °C (non-depletion conditions). Cells were pulse-labeled and processed as described under "Materials and Methods." Lpp and BRP were immunoprecipitated with anti-HA antiserum and OmpA with OmpA anti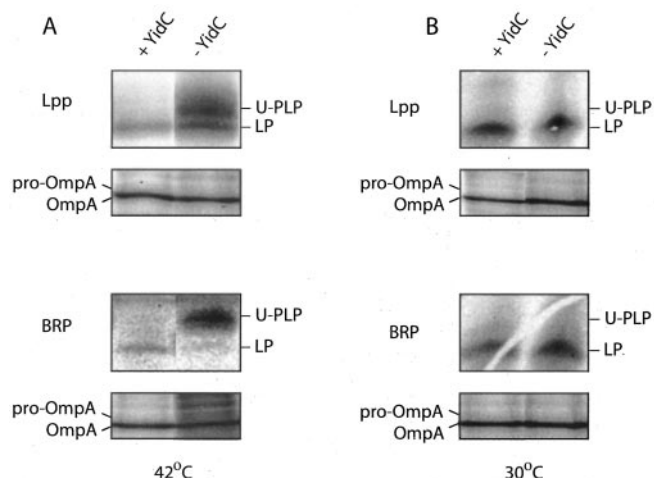serum. OmpA processing was not affected upon YidC depletion. YidC depletion was monitored by means of Western blotting and resulted, as expected, in a strong PspA response (Ref. 56 and results not shown).

*Nascent Lpp and BRP Synthesized in Vitro Cross-link to Ffh, SecA, SecY, and YidC*—To study the targeting and translocation of Lpp and BRP in more detail, we have used an *in vitro* translation/photo cross-linking approach. In this assay, the interactions of nascent (ribosome-associated) polypeptides with cytosolic and membrane components are fixed and analyzed. [$^{35}$S]Methionine-radiolabeled nascent chains of Lpp and BRP were synthesized in an *E. coli* cell-free extract from truncated mRNA to a length of 55 amino acids. Assuming that the ribosome covers ~35 amino acids, the Lpp and BRP signal sequences are expected to be exposed just outside the ribosome (37). To specifically probe the molecular environment of the

signal sequence in the nascent Lpp and BRP species, a single amber stop codon (TAG) was introduced in the center of the hydrophobic core in the Lpp (position 11) and BRP (position 10) signal sequences (Fig. 1). The amber stop codons were suppressed during the *in vitro* translation by addition of (Tmd-)Phe-tRNA$^{sup}$, an amber suppressor tRNA that is amino-acylated with the photo cross-linker (Tmd)Phe. In all constructs, the TAGs were efficiently suppressed by (Tmd)Phe-tRNA$^{sup}$ (data not shown). Purified inverted IMVs were added from the start of the translation reaction to allow cotranslational membrane targeting and interaction of the translation intermediates with the membrane. After the translation/insertion reaction, one half of each sample was irradiated with UV light to induce cross-linking; the other half was kept in the dark to serve as a control. The samples were extracted with carbonate to separate soluble and peripherally membrane-associated material from membrane-integrated components. Cross-linking partners were identified by immunoprecipitation. Without UV irradiation, no cross-linking products were detected using both Lpp and BRP nascent chains (data not shown). Upon translation but prior to UV irradiation, the samples were divided in two, and EDTA was added to one aliquot to provoke the release of the nascent chains from the ribosome. This allowed us to assess the importance of the context of the ribosome for cross-linking to the truncated Lpp and BRP species. EDTA has been shown to disassemble ribosomes (37).

Both nascent Lpp and BRP were efficiently targeted to the IMVs, judging from the relatively high (~50%) carbonate resistance. When the carbonate supernatant of UV-irradiated samples was analyzed, Lpp nascent chains were shown to cross-link Ffh, albeit inefficiently (Fig. 5*A*, *lane 3*), and the chaperone trigger factor (*TF*, *lane 4*). The ~30-kDa cross-linking adducts represented cross-linking to a breakdown product of Ffh, as observed before (39).

Cross-linking to both Ffh and TF appeared dependent on the context of the ribosome (*lanes 8, 9*) consistent with earlier studies (39, 40). In contrast, cross-linking to SecA was hardly detectable unless the nascent chains were released from the ribosomes prior to cross-linking (*lanes 7, 10*). Strong cross-linking specific for the ribosome-associated Lpp was observed to the ribosomal proteins L23 and L29 (*lanes 5, 6*). L23 and L29 are located near the exit site of the large ribosomal tunnel that runs from the peptidyl transferase center to the surface of the large ribosomal subunit (41). In the carbonate pellet, cross-linking to SecY and (weakly) to YidC was observed that appeared dependent on the ribosomal context (*lanes 13, 14, 16, 17*), again consistent with earlier studies (5, 37). In addition, in the pellet fractions SecA cross-linking was detectable only upon release of nascent Lpp from the ribosome. Together, the data suggest that nascent Lpp leaves the ribosome via the major exit tunnel near L23 and L29. Most likely, the signal sequence of a small fraction of nascent Lpp contacts the SRP. However, the majority of nascent Lpp is close to TF. Consistent with this explanation, both the SRP and TF dock near L23/L29 on the ribosome, probably in a mutually exclusive manner. Upon forced release of the nascent chains from the ribosome, the signal peptide loses contact with L23, L29, TF, and Ffh and is free to bind SecA, part of which is carbonate-resistant. Nascent Lpp is primarily targeted to SecY but also contacts YidC.

Qualitatively, very similar results were obtained when nascent BRP was used instead of Lpp (Fig. 5*B*). However, cross-linking to Ffh appeared much more prominent at the expense of cross-linking to TF (*lanes 1, 3, 7*), especially when considering the small amount of SRP present in cells as compared with TF (42, 43). Upon treatment with EDTA, cross-linking to Ffh was no longer detectable (*lane 8*), but another unknown factor
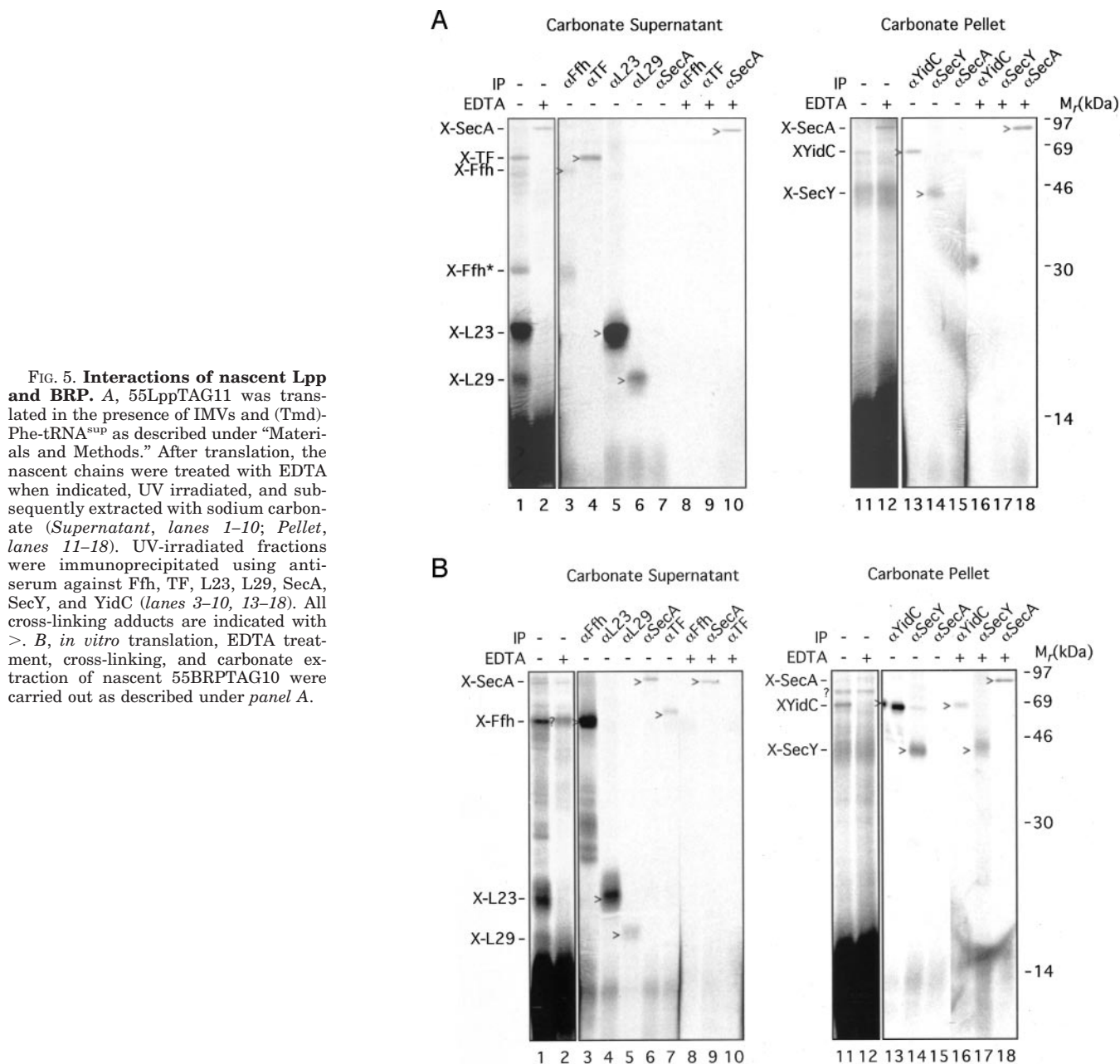
*BRP and LPP Targeting and Translocation*

FIG. 5. **Interactions of nascent Lpp and BRP.** *A*, 55LppTAG11 was translated in the presence of IMVs and (Tmd)-Phe-tRNA[sup] as described under "Materials and Methods." After translation, the nascent chains were treated with EDTA when indicated, UV irradiated, and subsequently extracted with sodium carbonate (*Supernatant*, *lanes 1–10*; *Pellet*, *lanes 11–18*). UV-irradiated fractions were immunoprecipitated using antiserum against Ffh, TF, L23, L29, SecA, SecY, and YidC (*lanes 3–10, 13–18*). All cross-linking adducts are indicated with >. *B*, *in vitro* translation, EDTA treatment, cross-linking, and carbonate extraction of nascent 55BRPTAG10 were carried out as described under *panel A*.

of about the same molecular mass (~50 kDa) was cross-linked (*lane 2*). Other notable differences were that ribosome-associated BRP also detectably cross-linked SecA (*lane 1*, *6*), and there was stronger cross-linking of targeted nascent BRP to YidC (*lane 11*). Finally, a cross-linking adduct of 75 kDa was observed in the carbonate pellet fractions both before and after release of nascent BRP from the ribosome, which remains to be identified (*lanes 11*, *12*). The more prominent contacts with the SRP and YidC suggest a more important role for these factors in the targeting and translocation of BRP, corroborating the *in vivo* data.

### DISCUSSION

Here, we have studied in *E. coli* the targeting and translocation of two secretory lipoproteins, Lpp and BRP, using a combined *in vivo* and *in vitro* approach. Surprisingly, the signal peptides of both nascent BRP and, to a lesser extent, Lpp show cross-linking to the targeting factor SRP and to the Sec-associated YidC, which are thought to function in the membrane targeting and integration of integral IMPs. Consistent with

these *in vitro* results, BRP and, to a lesser extent, Lpp depend on the presence of the SRP and YidC for efficient targeting to and translocation across the inner membrane *in vivo*.

*In vitro*, Lpp and BRP nascent chains with a length of 55 amino acids, carrying a UV-inducible cross-linker in the middle of the signal sequence, are also cross-linked to the ribosomal components L23 and L29 and to the ribosome-associated chaperone TF. L23 and L29 are located near the exit of the presumed ribosomal tunnel (41) and have been cross-linked to short nascent chains of other origin before (39, 44). L23 has recently been shown to function as an attachment site for both TF and SRP (39, 45, 46). Cross-link studies have identified TF as the first chaperone to interact generically with nascent polypeptides (47) unless they carry a particularly hydrophobic targeting signal that has a high affinity for the SRP (39, 40, 48). The mechanism that underlies the interplay between TF and SRP at the nascent chain exit site and how this interplay influences the mode of membrane targeting and insertion of a particular nascent protein are still unresolved issues (39, 44).

The SRP is primarily used for the targeting of IMPs that are thought to benefit from a cotranslational insertion mechanism to prevent aggregation of hydrophobic domains in the cytoplasm. Then why does the SRP appear to play such an important role in the targeting of the secretory lipoprotein BRP? First, the BRP signal sequence is very hydrophobic, more hydrophobic than many signal anchor sequences of SRP-dependent IMPs and prone to aggregation if unprotected. Interaction of the signal sequence with the SRP and cotranslational targeting of BRP may be the best way to prevent uncontrolled insertion of the hydrophobic BRP signal sequence into the inner membrane and aggregation of BRP in the cytoplasm. Recently, it has been suggested that basic amino acids in the N region of a signal sequence contribute to SRP binding, probably through the formation of salt bridges between the 4.5 S RNA and positively charged amino acids in the N region (49). Strikingly, the BRP signal sequence has 3 lysines in its N region, which may enhance even more the affinity of the already very hydrophobic BRP signal sequence for the SRP. These features may compensate for the relatively short time window in which the SRP can interact with nascent BRP, given that the BRP is a very small protein. It should be noted that the BRP signal peptide is peculiar in the sense that it is stable in the inner membrane, whereas other cleaved signal peptides are rapidly degraded upon cleavage from the precursor protein. Apparently, the BRP signal peptide is recognized as a signal anchor sequence of an IMP: it binds the SRP in the cytosol, inserts at the Sec translocon, and is subsequently transferred to the lipid bilayer as a stably folded unit assisted by YidC (see below).

The signal sequence of Lpp is not very hydrophobic, and its N region contains only one positively charged amino acid. However, it does show (weak) SRP cross-linking and SRP dependence *in vivo*. There are recent precedents of relatively non-hydrophobic signal peptides that are yet able to funnel passenger proteins into the SRP pathway (50). Therefore, it is not unlikely that there are yet unknown features of a signal sequence that can provoke SRP binding. There may be an important biological reason for a preference of Lpp for the SRP-targeting pathway. Lpp is the most abundant protein in *E. coli* and, therefore, highly expressed. When the mature part of Lpp is expressed in the cytoplasm it forms stable trimers (13). It is conceivable that cotranslational targeting of Lpp via the SRP pathway prevents the formation of Lpp trimers in the cytoplasm, which would make it incompetent for translocation. The partial effect of SRP depletion suggests that Lpp can also travel via SecB. The lack of SecB did not significantly affect the kinetics of Lpp translocation (32).[3] However, pre-Lpp could be detected in cytosolic aggregates isolated from a SecB null strain.[3] A similar flexibility in targeting has recently been demonstrated for the autotransporter Hbp (9). Therefore, it is not unlikely that when the capacity of the SRP pathway is insufficient, the SecB pathway gets a more prominent role in the targeting of Lpp and, perhaps, also BRP. Unfortunately, we have not succeeded in studying the targeting of Lpp and BRP in an Ffh depletion/SecB null mutant background. Therefore, other modes of targeting, like spontaneous or mRNA targeting, cannot be excluded at present (3, 51).

Cross-linking of Lpp and BRP nascent chains to SecA and SecY and the almost completely blocked maturation of both lipoproteins under SecA and SecE depletion conditions clearly show that both Lpp and BRP are translocated by the Sec translocase across the inner membrane, corroborating previous *in vivo* studies (30–33, 52). Surprisingly, both BRP and, to some extent, Lpp nascent chains are cross-linked to YidC, and

*in vivo* depletion of YidC affects the maturation of BRP and Lpp. In the context of the Sec translocon, YidC is considered to facilitate the transfer of TMs of IMPs from the Sec translocase into the lipid bilayer without being essential for this process. The role of YidC in the targeting/translocation of Lpp and BRP is enigmatic. It is possible that YidC facilitates the lateral movement of the Lpp and BRP signal sequences into the lipid bilayer or that YidC chaperones Lpp and BRP to the SPaseII/Lol system. It is also possible that there is a direct connection between SRP dependence and YidC dependence. It has been shown that the chloroplast homologues of SRP and YidC participate in targeting complexes (53). Therefore, it cannot be excluded that YidC plays a role in the SRP cycle, perhaps in the reception of the SRP or FtsY. Interestingly, membranes isolated from cells depleted of YidC show decreased levels of the lipoprotein CyoA, which in contrast to Lpp and BRP is an integral IMP (54, 55). This may point to a more general and not yet understood role of YidC in the targeting/translocation of lipoproteins. In conclusion, our results indicate that the SRP/Sec/YidC pathway is used by the secretory lipoproteins Lpp and BRP.

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