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Early events in preprotein recognition in *E.coli*: interaction of SRP and trigger factor with nascent polypeptides

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In *Escherichia coli*, components of a signal recognition particle (SRP) and its receptor have been identified which appear to be essential for efficient translocation of several proteins. In this study we use cross-linking to demonstate that *E.coli* SRP interacts with a variety of nascent presecretory proteins and integral inner membrane proteins. Evidence is presented that the interaction is correlated with the hydrophobicity of the core region of the signal sequence and thereby with its ability to promote transport *in vivo*. A second *E.coli* component, which is identified as trigger factor, can be efficiently cross-linked to all tested nascent chains derived from both secreted and cytosolic proteins. We propose that SRP and trigger factor act as secretion-specific and general molecular chaperone respectively, early in protein synthesis.

Keywords: *E.coli*/protein targeting/signal recognition particle/signal sequence/trigger factor

Introduction

The majority of secreted and membrane proteins in both prokaryotic and eukaryotic cells are synthesized as a precursor protein with an N-terminal signal sequence that is cleaved during insertion into the cytoplasmic and endoplasmic reticulum (ER) membrane respectively. Signal sequences do not exhibit sequence similarity but share common structural features. Most are 20–30 residues long and are composed of a positively charged N-terminal domain, a hydrophobic core region and a polar C-terminal domain encompassing the signal peptidase cleavage site (von Heijne, 1988; Izard and Kendall, 1994). Moreover, signal sequences are usually interchangeable between prokaryotic and eukaryotic cells suggesting a general similarity of all signal sequences and a common role in protein targeting and membrane translocation (Hartl and Wiedmann, 1993; High and Stirling, 1993; Luirink and Dobberstein, 1994; Wolin, 1994).

In eukaryotic systems, the signal sequence of nascent (ribosome-associated) preproteins interacts with the 54 kDa protein of the signal recognition particle (SRP), a ribonucleoprotein complex consisting of a 7S RNA and six polypeptides (Walter and Johnson, 1994; Lütcke, 1995). This interaction is believed to keep the nascent chain in a translocation competent conformation until the SRP contacts its receptor ('docking protein') at the ER membrane, prior to the actual membrane insertion process.

In *Escherichia coli*, the role of the signal sequence in targeting of preproteins to the cytoplasmic membrane is less well understood. Components of the so-called general secretory pathway (GSP) have been implicated in recognition of the signal sequence in the cytosol (reviewed in Pugsley, 1993; Arkowitz and Bassilana, 1994). The secretion specific chaperone SecB was found to interact with a small subset of nascent preproteins (Kumamoto and Francetic, 1993), but whether it is at any stage in direct contact with the signal sequence is still a matter of dispute. Chemical cross-linking studies have indicated that the ATPase SecA which is believed to function as an adapter between SecB and the translocon in the membrane, binds to the N-terminal region of the signal sequence by electrostatic interaction (Akita *et al.*, 1990). Furthermore, functional signal sequences were shown to inhibit the ATPase activity of SecA which accompanies the translocation reaction (Cunningham and Wickner, 1989). A variety of biophysical techniques has been employed to demonstrate that signal sequences are also able to interact directly with lipid bilayers (Izard and Kendall, 1994).

Recently, evidence has accumulated that, in addition to the GSP, an alternative targeting pathway exists in *E.coli* that involves an SRP (Hartl and Wiedmann, 1993; Luirink and Dobberstein, 1994; Wolin, 1994). Components were identified in *E.coli* that exhibit strong sequence similarity to SRP54, SRP7S RNA and the α-subunit of the SRP-receptor (Poritz *et al.*, 1988; Bernstein *et al.*, 1989; Römisch *et al.*, 1989). *Escherichia coli* P48 (SRP54 homolog) and 4.5S RNA (7S RNA homolog) form a ribonucleoprotein particle (Poritz *et al.*, 1990; Ribes *et al.*, 1990) that could be specifically cross-linked to the signal sequence of nascent preprolactin in crude lysates and compete the interaction of mammalian SRP (Luirink *et al.*, 1992). Furthermore, P48 could replace SRP54 in a reconstituted mammalian SRP without affecting signal sequence recognition (Bernstein *et al.*, 1993). Finally, P48–4.5S RNA complex and FtsY (SRP-receptor homolog) were shown to interact *in vitro* (Miller *et al.*, 1994) and to be essential *in vivo* for efficient membrane translocation of several secreted proteins (Phillips and Silhavy, 1992; Luirink *et al.*, 1994). Notably, proteins that are secreted independently of SecB were most strongly affected by depletion of P48, consistent with the idea of parallel targeting pathways with partly overlapping substrate specificity (Phillips and Silhavy, 1992).

In this study, we use a photo cross-linking approach to get more insight into the early stages in the recognition of *E.coli* presecretory and membrane proteins and the

molecular environment of nascent *E.coli* polypeptide chains in general. Evidence is presented that P48 interacts with a variety of nascent presecretory proteins (both of the SecB-dependent and -independent type) and integral inner membrane proteins. The interaction is found to be correlated with the hydrophobicity of the core region of the signal peptide and thereby with the ability of the signal peptide to promote translocation *in vivo*. Surprisingly, most nascent chains, derived from both secreted and non-secreted proteins, can be cross-linked to a second *E.coli* component identified as trigger factor (TF). TF has originally been implicated in protein translocation based on its ability to form isolable complexes with purified proOmpA (Crooke and Wickner, 1987) which stimulated the translocation of proOmpA into inverted inner membrane vesicles (Crooke and Wickner, 1987; Crooke *et al.*, 1988a,b). However, cells depleted of trigger factor did not exhibit pleiotropic secretion defects arguing against a role in secretion *in vivo* (Guthrie and Wickner, 1990). Our results suggest that trigger factor has a general ability to bind to short nascent polypeptides and we propose that it may function as a chaperone early in protein synthesis.

Results

P48 interacts with nascent E.coli presecretory proteins

We used light-induced cross-linking (Figure 1) to identify proteins in an *E.coli* lysate that interact with different nascent presecretory protein chains of *E.coli* origin. Genes encoding various model proteins (Figure 2) were subcloned into transcription vectors to facilitate the preparation of large quantities of messenger RNA. Truncated messenger RNAs were prepared and translated to generate ribosome–nascent chain complexes. The length of the nascent chain was chosen to obtain an optimal exposure of the signal sequence considering that 30–40 amino acids are sequestered within the ribosome (Walter and Blobel, 1981; Meyer *et al.*, 1982; Mothes *et al.*, 1994). Translation was carried out in the presence of [^{35}S]methionine to label the polypeptide and in the presence of 4-(3-trifluoromethyl-diazirino)benzoic acid-modified (TDBA) lysyl-tRNA to incorporate photo reactive cross-linking groups in the nascent chain at the position of the lysine residues. Since yeast TDBA lysyl-tRNA is not accepted in *E.coli* translation, a wheat germ translation system was used (Luirink *et al.*, 1992). Ribosome–nascent chain complexes were purified over a high salt sucrose cushion to remove all associated wheat germ components, incubated with crude *E.coli* cell lysate and irradiated with UV light to induce cross-linking.

Upon UV irradiation, nascent PhoE, an outer membrane porin which is dependent on SecB for efficient translocation across the inner membrane (de Cock *et al.*, 1992), gave rise to cross-linking products of 58 and 65 kDa (Figure 3A, lanes 1 and 2). The 58 kDa cross-linked product (X-P48) consists of nascent PhoE (10 kDa) and the SRP constituent P48, as could be demonstrated by immunoprecipitation of the product using antisera directed against P48 (Figure 3A, lane 4) and PhoE (not shown) but not using a control antiserum (Figure 3A, lane 5). Furthermore, the amount of the 58 kDa product increased when a lysate from a strain that overproduces P48 was

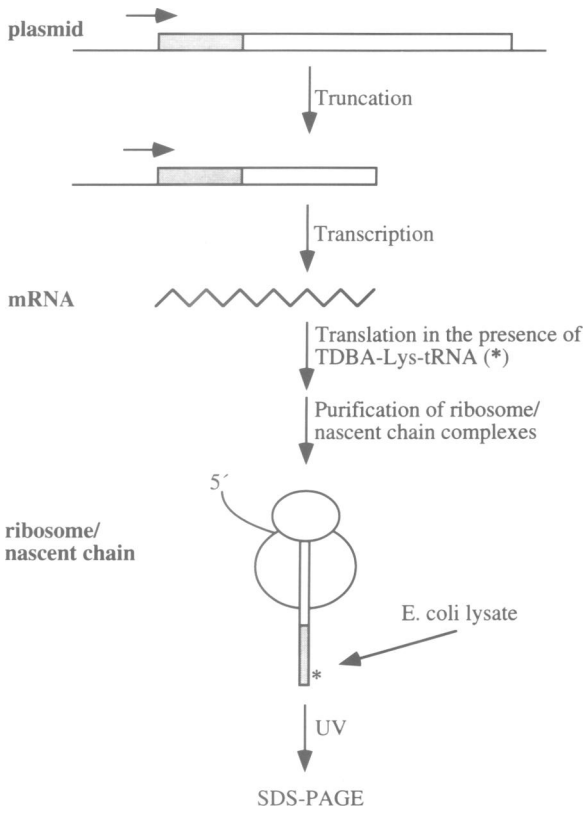

Fig. 1. Schematic representation of photo cross-linking assay. The assay is described in the text.

used (Figure 3A, lanes 6–10). The identity of the cross-linking partner in the 65 kDa product (X-P55) will be discussed below. Both X-P48 and X-P55 migrated as doublet bands which is seen more often in cross-linked nascent chains (see also below). This may be caused by some heterogeneity in nascent chain length or differences in the modified lysine residue from which cross-linking occurred. In addition, P48 cross-linked from WT lysate to nascent LamB (data not shown), another SecB-dependent outer membrane porin (Kumamoto and Beckwith, 1985).

To investigate the specificity of P48 binding for functional signal sequences, a PhoE mutant lacking the signal sequence (PhoE-Δss; Figure 2) was used as a substrate for cross-linking. Using WT lysate, no cross-linking to P48 was observed (Figure 3B, lanes 1–5). Since nascent PhoE-Δss is somewhat shorter than nascent PhoE-WT, a shorter PhoE nascent chain containing a functional signal sequence (PhoE-trunc1; Figure 2) was prepared to serve as a control substrate. Strong cross-linking of PhoE-trunc1 nascent chain to P48 was observed (Figure 3C). Taken together, these data indicate that P48 binds specifically to the signal sequence of nascent PhoE. Upon overproduction, P48 was found cross-linked to nascent PhoE-Δss (Fig 3B, lanes 6–10) as well as to the nascent non-signal sequence bearing proteins chloramphenicol acetyl transferase (CAT) (data not shown) and firefly luciferase (ffLuc) (see below).

To study whether P48 has a general affinity for signal sequences, two other nascent presecretory proteins were tested in the same experimental set-up: murein lipoprotein (Lpp), the most abundant *E.coli* outer membrane protein and alkaline phosphatase (PhoA), a periplasmic enzyme.

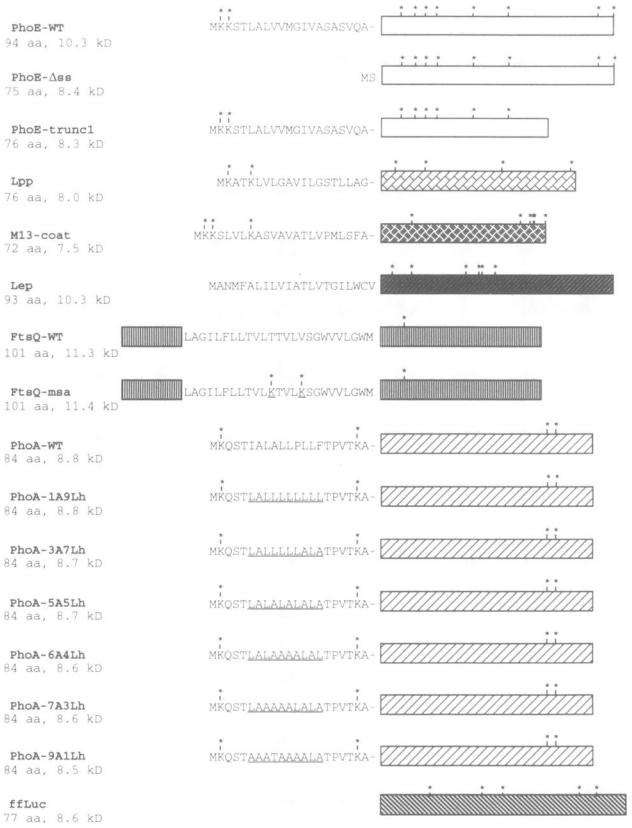

Fig. 2. Outline of the nascent polypeptides used in this study. The length and predicted molecular weight of the nascent polypeptides are indicated. The amino acid sequence of the signal peptide and signal anchor domains are shown (substituted amino acids are underlined). The mature regions are presented by boxes separated by a dash from cleavable signal sequences. The positions of the lysine residues which may serve as cross-linking sites are indicated by asterisks.

Both Lpp and PhoA do not depend on the cytosolic chaperone SecB for efficient sorting (Kumamoto and Beckwith, 1985; Watanabe *et al.*, 1988). Pre-Lpp is cleaved by the lipoprotein-specific signal peptidase II which recognizes a specific tetrapeptide at the extreme C-terminus of the signal sequence. The cross-linking patterns of Lpp and PhoA (Figure 3D and E) did not differ qualitatively from those obtained with PhoE-WT, the amount of P48 cross-linked from WT lysate to nascent Lpp being relatively low compared with the amount of cross-linked P55 (Figure 3D, lanes 2–4).

Taken together, these results suggest that P48 has a general ability to bind to exposed signal sequences of short nascent chains, derived from either SecB-dependent or -independent preproteins.

P48 interacts with nascent E.coli inner membrane proteins

In addition to nascent presecretory proteins, nascent chains of both type I and II signal anchor proteins can be cross-linked to SRP54 of mammalian SRP (reviewed in High and Dobberstein, 1992). Having shown that P48 interacts with secreted proteins, we investigated the interaction of P48 and three nascent *E.coli* inner membrane proteins (Figure 2) using the cross-linking approach described above.

M13 procoat protein, a simple class I membrane protein which contains a cleavable signal sequence and inserts independently from SecB, SecA and SecY (Silver *et al.*, 1981; Watts *et al.*, 1981), was cross-linked to P48 from WT lysate (Figure 4A, lanes 1–5) and P48-enriched lysate (Figure 4A, lanes 6–10).

Leader peptidase I (Lep), a polytopic membrane protein with an uncleaved signal sequence which inserts independently from the Sec machinery (Lee *et al.*, 1992), was also efficiently cross-linked to P48 from WT lysate (Figure 4B, lanes 1–5) and P48-enriched lysate (Figure 4B, lanes 6–10). Since the signal sequence does not contain lysine residues, cross-linking is probably taking place from one of two modified lysine residues present just C-terminal from the signal sequence and not buried inside the ribosome (Mothes *et al.*, 1994).

FtsQ (FtsQ-WT), a simple class II membrane protein with a short N-terminal cytoplasmic tail (Carson *et al.*, 1991), was synthesized as a slightly longer nascent chain (101 residues; Figure 2) than usual to allow optimal exposure of the signal anchor (SA) domain. Weak cross-linking of FtsQ-WT to P48 was observed when either the WT lysate or the P48-enriched lysate was used (data not shown). We suspected this to be due to the absence of lysine residues in the SA domain which leaves only one lysine residue, 10 residues C-terminal from the SA domain as a potential cross-linking site. To investigate this possibility, two lysine residues were introduced in the centre of the SA domain by site directed mutagenesis (FtsQ-msa; Figure 2). In comparison with FtsQ-WT, FtsQ-msa was more efficiently cross-linked to P48 from WT lysate (Figure 4C, lanes 1–5) and P48-enriched lysate (Figure 4C, lanes 6–10) indicating that its SA domain interacts with P48.

Taken together, the results suggest that P48 interacts with signal sequences (both cleavable and uncleavable) and SA domains of *E.coli* integral inner membrane proteins analogous to its mammalian counterpart SRP54.

Interaction of P48 correlates with the hydrophobicity of the signal sequence core region

The hydrophobic core region is the most obvious feature of both cleaved and uncleaved signal sequences (Izard and Kendall, 1994). Although some variation in core region length exists, there are limits to the minimum and maximum number of hydrophobic residues that constitute a functional signal sequence. Based on secondary structure predictions it has been proposed that the C-terminal, methionine rich M-domain of SRP54 contains four amphipathic helices which form a hydrophobic binding pocket to accommodate a variety of signal sequences of sufficient hydrophobicity (Bernstein *et al.*, 1989).

To investigate whether P48, which contains a highly homologous M-domain, recognizes the hydrophobic core region, we made use of a systematic series of mutants in which the core segment of the *E.coli* PhoA signal sequence has been replaced by a stretch of 10 leucine (Leu) and alanine (Ala) residues (Doud *et al.*, 1993; see Figure 2). Thus, the composition of the core region varies from 9:1 to 1:9 in the ratio of Leu (more hydrophobic) to Ala (less hydrophobic) residues. Using *in vivo* precursor processing to quantify transport activity, a clear, non-linear dependency has been observed (Doud *et al.*, 1993). At ratios

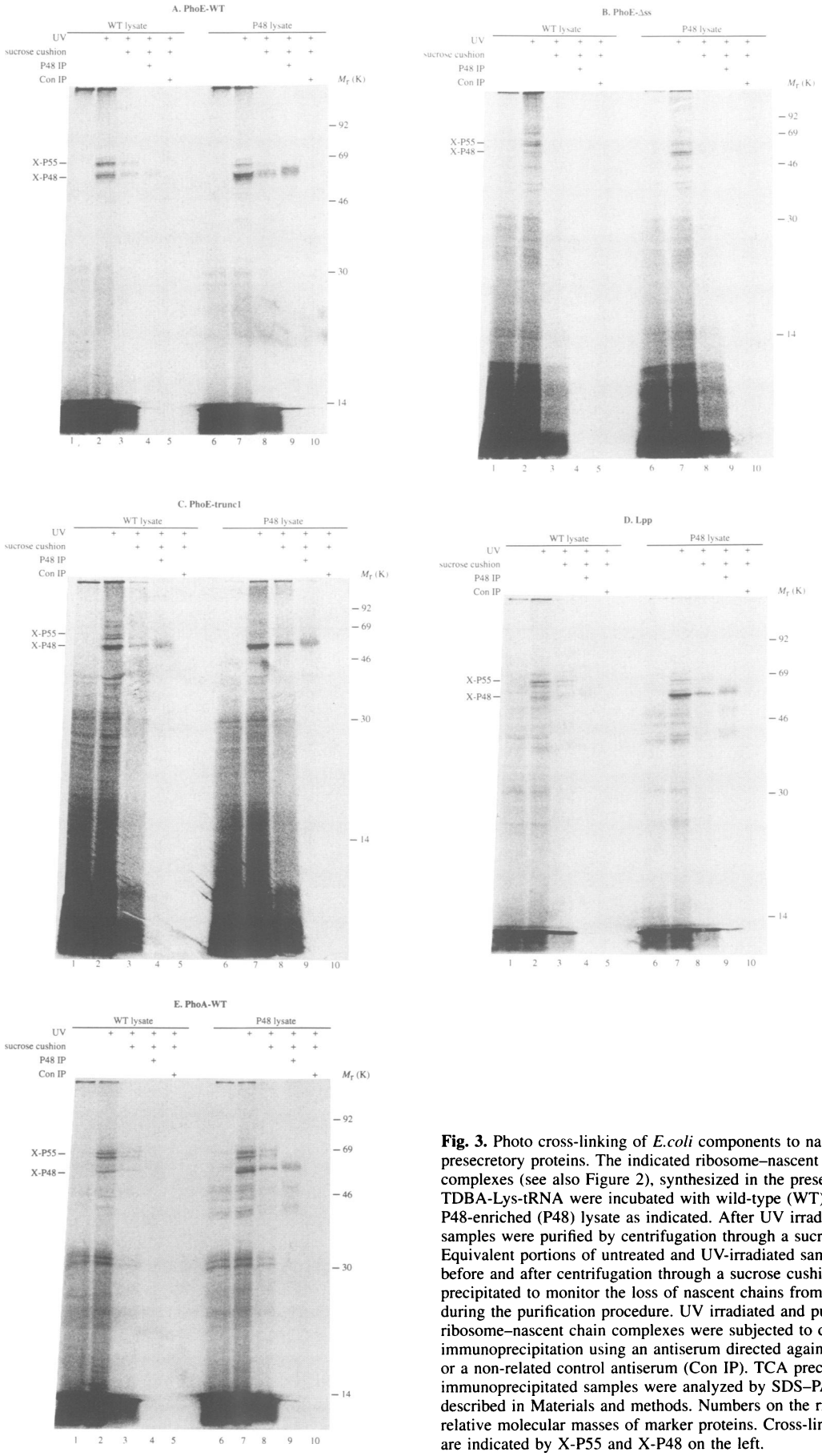

Fig. 3. Photo cross-linking of *E.coli* components to nascent presecretory proteins. The indicated ribosome–nascent chain complexes (see also Figure 2), synthesized in the presence of ε-TDBA-Lys-tRNA were incubated with wild-type (WT) *E.coli* lysate or P48-enriched (P48) lysate as indicated. After UV irradiation the samples were purified by centrifugation through a sucrose cushion. Equivalent portions of untreated and UV-irradiated samples (taken before and after centrifugation through a sucrose cushion) were TCA precipitated to monitor the loss of nascent chains from the ribosomes during the purification procedure. UV irradiated and purified ribosome–nascent chain complexes were subjected to denaturing immunoprecipitation using an antiserum directed against P48 (P48 IP) or a non-related control antiserum (Con IP). TCA precipitated and immunoprecipitated samples were analyzed by SDS–PAGE as described in Materials and methods. Numbers on the right indicate the relative molecular masses of marker proteins. Cross-linked products are indicated by X-P55 and X-P48 on the left.

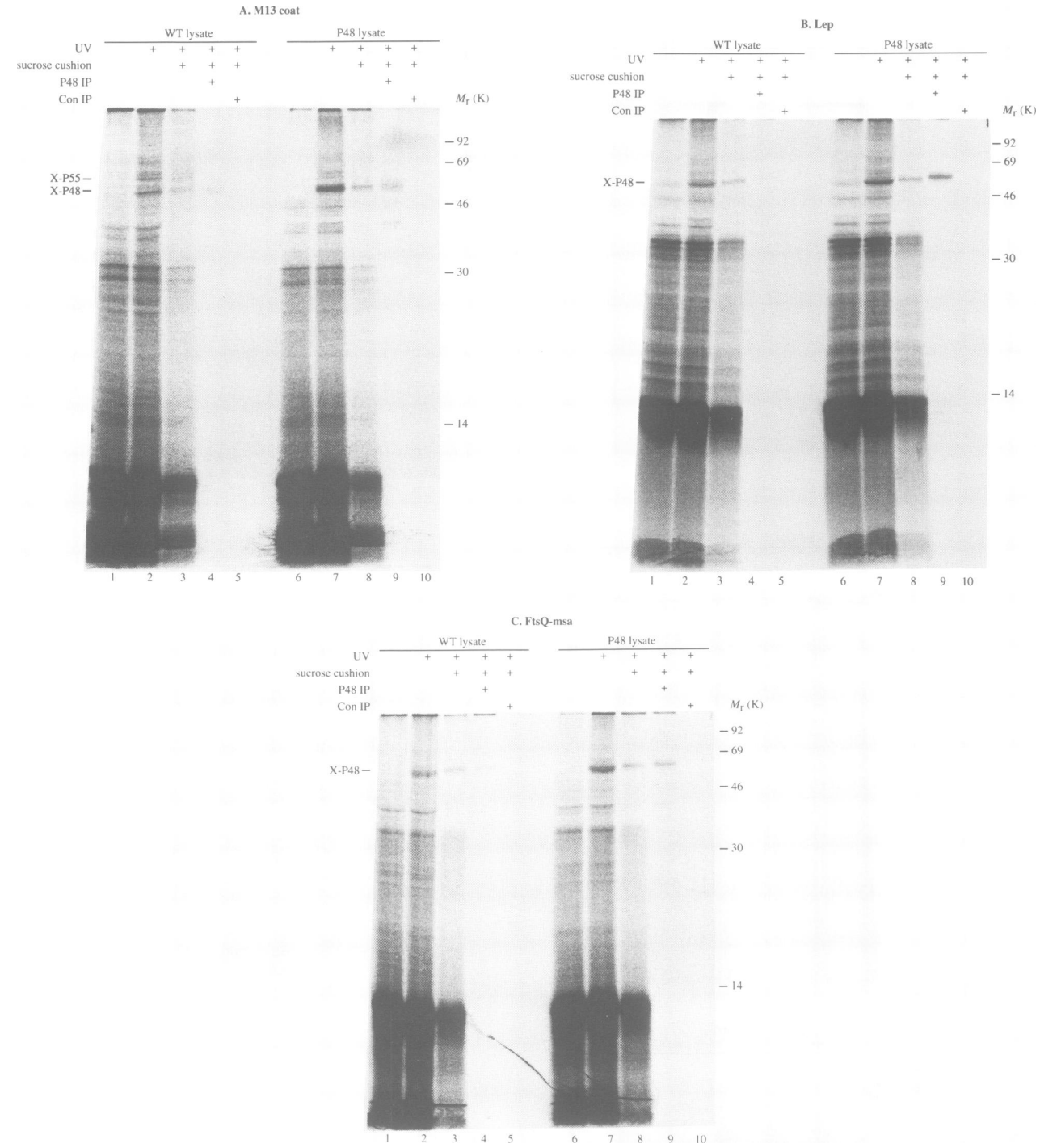

Fig. 4. Photo cross-linking of *E.coli* components to nascent inner membrane polypeptides. (For experimental details see the legend to Figure 3.)

of Leu to Ala of ⩾5:5 the signal sequence functions efficiently.

Nascent chains containing 84 residues of the mutants and PhoA-WT were prepared and tested for cross-linking to components from an *E.coli* lysate as described above. A strong, non-linear correlation was found between the hydrophobicity of the exposed mutant signal sequence and the amount of cross-linked P48 from WT lysate (Figure 5A, lanes 1–6; quantified in Figure 5B). PhoA-WT which contains a signal sequence of intermediary hydrophobicity showed intermediary cross-linking to P48 (Figure 5A, lane 7 and 5B). The same correlation was observed when the P48-enriched lysate was used (Figure 5A, lanes 8–14). In this case however, even the mutant with the most hydrophilic signal sequence (Figure 5A, lane 13) cross-linked significant amounts of P48 resembling the results obtained with PhoE-Δss (Figure 3B, lanes 6–10). Apparently, P48 can be cross-linked to all nascent chains regardless of whether or not they harbor a functional signal sequence, provided that P48 is present in excess. Purified P48 and mammalian SRP also cross-linked preferentially to the more hydrophobic mutant signal sequences (results not shown) indicating that *E.coli* and mammalian SRP share comparable binding specificity.

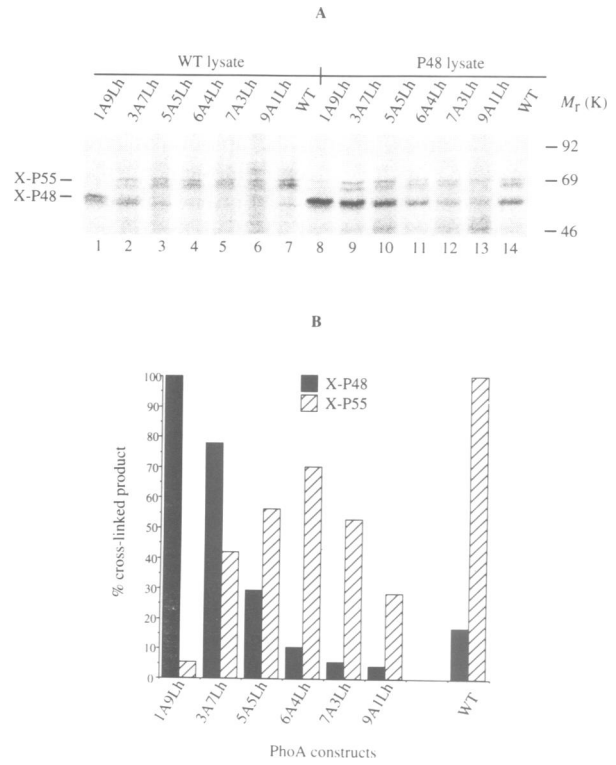

Fig. 5. Photo cross-linking of *E.coli* components to nascent PhoA and mutant derivatives. (**A**) SDS–PAGE analysis. The nascent chains used are indicated on top of the lanes (see Figure 2). For details concerning the experimental set-up, see the legend to Figure 3 except that all samples were TCA precipitated after UV-irradiation. Because varying amounts of nascent chains were synthesized, as assessed by phosphor image quantification, corresponding amounts were used for photo cross-linking. (**B**) Quantitation of data presented in (A), lanes 1–7. The ratio of the amount of cross-linked nascent chains (X-P48 and X-P55) to the total amount of nascent chains was calculated for each construct and related to the maximal values obtained for X-P48 and X-P55 which were set at 100%.

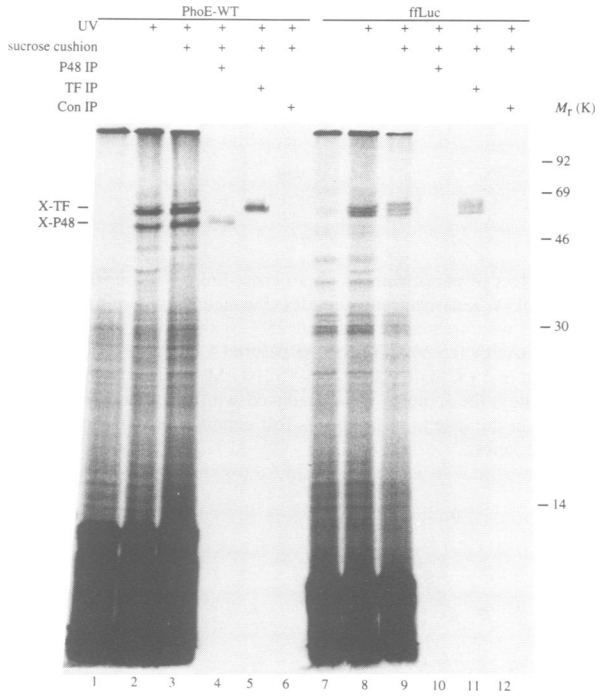

Fig. 6. Photo cross-linking of *E.coli* components to nascent PhoE-WT and ffLuc polypeptides. Denaturing immunoprecipitations were carried out using antisera directed against P48 (P48 IP), TF (TF IP) or a non-related control antiserum (Con IP). For experimental details see the legend to Figure 3.

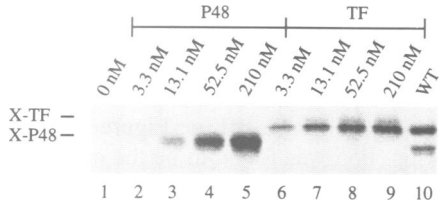

Fig. 7. Photo cross-linking of purified P48 and TF to nascent PhoE-WT polypeptides. Samples were treated as indicated in the legend to Figure 5. The concentration of pure protein used is indicated at the top of the lanes. Cross-linking in WT lysate is included as a control.

Taken together, the results suggest that the hydrophobicity of the core region is decisive for recognition of signal sequences by the SRP.

P55 is trigger factor which interacts with nascent presecretory proteins

In the studies described above, an *E.coli* component of ~55 kDa (P55) was found to be cross-linked to all nascent presecretory and membrane proteins including PhoE-Δss and the PhoA mutant series but with the exception of Lep and FtsQ (Figures 3–5). To identify P55, antibodies raised against putative interacting components (Sec-proteins, chaperones) were tested for their ability to immunoprecipitate X-P55 after cross-linking of nascent PhoE-WT in the presence of WT lysate. Using this approach P55 was identified as trigger factor (TF; Figure 6, lane 1–6) which has a predicted molecular mass of 48 kDa based on sequence analysis (Guthrie and Wickner, 1990) whereas the apparent mass in SDS–PAGE is 60 kDa (Crooke and Wickner, 1987). To obtain further proof that TF interacts with nascent PhoE, pure TF was added at different concentrations prior to cross-linking instead of lysate. Figure 7 shows that even very low (subphysiological) concentrations of TF (lane 6, 3.3 nM) still result in clearly detectable cross-linked products with nascent PhoE comigrating with X-P55. We conclude that TF interacts very efficiently with nascent pre-PhoE.

P48 only interacts with ribosome-associated nascent polypeptides (Luirink *et al.*, 1992). To investigate whether this is also true for TF, ribosome-nascent PhoE-WT complexes were disrupted with puromycin in high salt buffer prior to the addition of purified TF. TF cross-linking was prevented by the combined high salt/puromycin treatment (Figure 8, lanes 2 and 6) similar to the effect on the cross-linking of P48 (Figure 8, lanes 1 and 5; Luirink *et al.*, 1992). High salt treatment alone already resulted in a decrease in cross-linking of both P48 and TF (Figure 8, lanes 3 and 4), an effect which was also observed when using WT lysate instead of purified proteins though to a lesser extent (data not shown; Luirink *et al.*, 1992). We conclude that TF only binds polypeptides in the context of the ribosome.

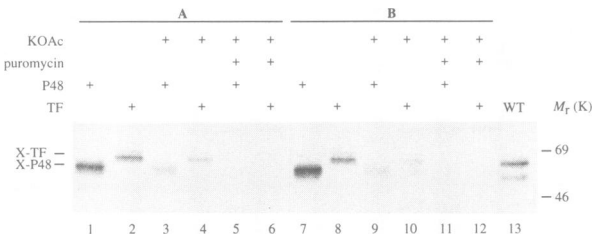

Fig. 8. Effect of puromycin on photo cross-linking of purified P48 or TF to PhoE-WT nascent polypeptides. Purified ribosome–nascent chain complexes were treated for 5 min at 25°C with 2 mM puromycin and 0.5 M KOAc or 0.5 M KOAc alone before (**A**) or after (**B**) a 5 min incubation at 25°C with purified P48 (210 nM) or TF (70 nM). Subsequently, the samples were irradiated with UV light and treated as indicated in the legend to Figure 5. Test conditions are indicated on top of the lanes.

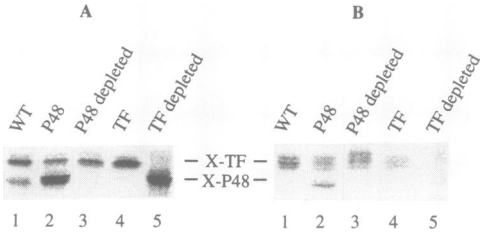

Fig. 9. Photo cross-linking from lysates enriched or depleted for P48 and TF to PhoE-WT (**A**) and ffLuc (**B**). Samples were treated as indicated in the legend to Figure 5. Used lysates are indicated on top of the lanes.

TF interacts with nascent non-signal sequence bearing proteins

P55 was also found cross-linked to nascent PhoE-Δss (Figure 3B). To investigate whether TF has a general ability to interact with non-signal sequence bearing proteins, short nascent firefly luciferase (ffLuc; Figure 2) was tested for cross-linking. P55 was found to be the major cross-linked component in WT lysate (Figure 6, lanes 7–9) and could be identified as TF by immunoprecipitation (Figure 6, lanes 11 and 12), whereas cross-linked P48 was not detected (Figure 6, lanes 7–10). Similar results were obtained using short nascent chains of CAT (data not shown) indicating that TF has a general affinity for short ribosome bound polypeptides.

Specificity of TF–nascent chain interaction

When P48-enriched lysate is used for cross-linking, the amount of TF cross-linked to nascent PhoE-WT, PhoE-trunc1, Lpp (Figure 3A, C and D) and M13 coat (Figure 4A) decreases, possibly due to competition of the two proteins for the same or overlapping sequences in the preprotein. To study this possibility in more detail, the amounts of P48 and TF in the lysates used for cross-linking to nascent PhoE-WT were varied (Figure 9). When lysate from P48 overproducing cells was tested for cross-linking, the amount of cross-linked TF decreased (Figure 9A, lanes 1 and 2; see also Figure 3A, lanes 2 and 7). Consistently, when purified P48 was titrated into P48-depleted lysate prior to cross-linking, the amount of cross-linked TF present in the lysate decreased in a concentration-dependent manner (data not shown). On the other hand, P48 depletion does not result in increased cross-linking of TF to nascent PhoE-WT (Figure 9A, lanes 1 and 3). The use of lysate from TF overproducing cells completely prevented cross-linking of P48 although cross-linking of TF was only slightly enhanced (Figure 9A, lane 4). Consistently, when purified TF was titrated into WT lysate prior to cross-linking, the amount of cross-linked P48 decreased in a concentration-dependent manner whereas cross-linking of TF was only slightly enhanced (data not shown). The use of TF-depleted lysate resulted in increased P48 cross-linking (Figure 9A, lane 5). Taken together, the results demonstrate that P48 and TF compete for cross-linking to nascent PhoE-WT. The reasons for the effects of increased levels of TF in the lysate (decreased cross-linking of P48, only slightly increased cross-linking of TF) are unclear at present. Possibly, TF competes directly with P48 for binding to nascent PhoE-WT but the interaction is not of sufficient affinity or proximity to allow cross-linking. Alternatively, TF may compete indirectly, for instance by inhibition of the interaction of P48 with ribosomes.

When using WT lysate, TF was found cross-linked to all nascent presecretory and non-signal sequence bearing proteins which were tested (Figures 3, 4A and 6). The lack of detectable TF cross-linking to the inner membrane proteins Lep and FtsQ-msa might be caused by the relatively strong hydrophobicity of their exposed SA sequences (Figure 4B and C). P48, which binds preferentially to hydrophobic sequences (see Figure 5A), might compete with TF for (partially overlapping) binding sites. This might also explain why PhoA mutants harboring a more hydrophobic signal sequence are less efficiently cross-linked to TF (Figure 5A). Consistent with this view, TF was found cross-linked to Lep when P48-depleted lysate or TF-enriched lysate was used (data not shown).

The interaction of TF with nascent proteins is reminiscent of the interaction of the eukaryotic nascent-polypeptide-associated complex (NAC) which binds to a variety of ribosome bound nascent chains of non-signal sequence bearing proteins early in translation (Wiedmann *et al.*, 1994). In the absence of NAC, SRP was found cross-linked to these polypeptides suggesting that NAC confers specificity to the interaction of SRP with the exposed signal sequence of nascent preproteins (Wiedmann *et al.*, 1994). To investigate whether this also holds true for TF, the levels of P48 and TF present in the lysates used for cross-linking to nascent ffLuc were varied as described above (Figure 9B). Notably, when lysate from a TF-depleted strain was used, TF cross-linking was diminished but not replaced by P48 cross-linking (Figure 9B, lane 5) indicating that TF does not exert a NAC-like activity. Cross-linking to TF was not increased when a TF-enriched lysate was used indicating that the interaction of TF with ffLuc is saturated in WT lysate (Figure 9B, lane 4). Remarkably, P48 could be cross-linked from a P48-enriched lysate with a concomitant slight decrease in TF cross-linking (Figure 9B, lane 2). Comparable results were obtained with PhoEΔss (Figure 3B, compare lanes 2 and 7). Apparently, overproduction of P48 results in its interaction with nascent, non-signal sequence bearing polypeptides.

Stability of the TF–nascent chain interaction

To determine the stability of the interaction between TF and nascent pre-PhoE, purified TF was allowed to bind

to nascent PhoE. Ribosome-nascent chain complexes were then treated with puromycin in high salt buffer or detergents prior to cross-linking. TF cross-linking was not detectable after puromycin/high salt treatment (Figure 8B, compare lanes 8 and 12) indicating that TF does not remain associated with released nascent PhoE similar to P48 (Figure 8B, compare lanes 8 and 12; Luirink *et al.*, 1992). High salt treatment alone reduced cross-linking of both P48 (Figure 8B, lane 9) and TF (Figure 8B, lane 10). Similar results were obtained when TF and P48 were cross-linked from WT lysate instead of using purified proteins although the interaction of both TF and P48 were more resistant to high salt alone under these conditions (data not shown) as observed before (Luirink *et al.*, 1992). Treatment of ribosome–nascent chain complexes with 1% NP40 or Triton X-100 did not affect cross-linking of either TF, P48 or mammalian SRP (data not shown). Thus, the stability of and requirements for interaction of TF with nascent PhoE are similar to those of P48 and mammalian SRP (High *et al.*, 1991b).

Discussion

To get more insight into the role of the SRP in the early stages of preprotein targeting in *E.coli* we have probed the environment of signal sequences of short nascent polypeptides by using a photo cross-linking approach. Our data show that *E.coli* P48, the SRP54 homolog, interacts specifically with a variety of nascent presecretory and membrane proteins early in their synthesis. Surprisingly, TF was found to have a general affinity for short nascent polypeptides.

Interactions with P48

Using WT *E.coli* lysate as a source for interacting components, P48 was preferentially cross-linked to all tested nascent presecretory and integral membrane proteins harboring a functional signal sequence or SA domain. Under the experimental conditions used, interaction appeared to be independent of any requirements of the preproteins for Sec factors for efficient membrane insertion and export *in vivo*. Thus, P48 interacted with nascent chains of preproteins that require SecB, SecA and SecY (PhoE and LamB), only SecA and SecY (PhoA and Lpp) or none of the Sec factors (M13 coat) for efficient membrane transport and insertion. Furthermore, uncleaved SA sequences both N-terminal (Lep) and internal (FtsQ) were efficiently cross-linked to P48. By using the same assay it has been shown previously that P48 interacts with the signal sequence of nascent preprolactin, a eukaryotic secreted protein (Luirink *et al.*, 1992). Thus, P48 appears to have a general affinity for nascent secretory and membrane proteins, as does its mammalian counterpart SRP54 (reviewed in Lütcke, 1995). When P48 is present in excess in the lysate, it is also cross-linked, albeit inefficiently, to nascent chains lacking functional signal sequences indicating that P48 has some affinity for nascent chains in general. A fraction of the overexpressed P48 is not assembled into SRP because the endogenous 4.5S RNA is not present in sufficient amounts and it may be that this free P48 has a more relaxed specificity. This possibility is currently being investigated. It should also be noted that cross-linking does not reflect the strength of binding observed. Thus, the cross-linking of P48 with proteins lacking a functional signal sequence may represent a low affinity interaction.

P48 was the only factor in WT lysate that cross-linked specifically to exposed signal sequences in our assay despite its very low abundance of ~50 copies per cell (Wikström and Björk, 1988 and data not shown). No other (more abundant) candidate interacting proteins, such as SecB and SecA, were detected even when the lysates used for cross-linking were derived from cells which strongly overproduce either of these proteins. Presumably, SecB which has been shown to interact co-translationally with a subset of secreted proteins (Kumamoto and Francetic, 1993), binds at a later stage in translation, probably to the mature region of the nascent polypeptide. These data argue against a strict and early separation in targeting pathways based on the exclusive binding of the SRP to a subset of signal peptides.

For mammalian SRP it has been suggested that the binding affinity may vary from signal peptide to signal peptide (Rapoport *et al.*, 1987). Furthermore, the time window in which the signal peptide is available for interaction *in vivo* is probably dependent on the rate of translation, the folding characteristics of the nascent protein and the interaction of cytosolic factors with other regions of the nascent chain. The cross-linking approach does not allow binding affinities to be determined and makes use of short nascent chains of fixed length which will effectively equalize the time window for interaction. Therefore, it is difficult to predict the proportion of nascent preproteins that interacts with P48 *in vivo*. However, in view of the strong and selective cross-linking of P48 and its low abundance in the cells, we consider it likely that binding of SRP is the first step in the targeting of many, if not all, secreted and membrane proteins in *E.coli*. In this respect it is of interest to note that depletion of P48 has an inhibitory effect on protein transport in general although transport of SecB independent proteins appeared to be most strongly affected (Phillips and Silhavy, 1992).

At present, we can only speculate about subsequent interactions of the growing nascent preprotein. A subset of proteins may bind SecB co-translationally at a later stage but it remains to be established whether simultaneous interaction of SRP and SecB with the nascent chain can occur or whether their binding is mutually exclusive. It is unclear whether SecA which has affinity for SecB and preproteins is able to interact co-translationally. Release of the SRP could be induced by these secondary interactions or by a direct interaction at the inner membrane between the SRP and FtsY, the *E.coli* homolog of the eukaryotic docking protein (Miller *et al.*, 1994; Luirink *et al.*, 1994).

What is the molecular basis of the specificity of signal peptide recognition? It has been proposed that the hydrophobic nature of the core region of the signal peptide is essential for its recognition by the SRP in both eukaryotes and prokaryotes. This hypothesis is supported by the lack of SRP cross-linking to a mutant preprolactin signal peptide in which the core region had been mutated at three positions, thereby lowering its overall hydrophobicity (Luirink *et al.*, 1992). We have been able to address the question of specificity more systematically by using a series of mutant PhoA derivatives in which the 10 residues

long core region has been replaced by a stretch of Leu (more hydrophobic side chain) and Ala (less hydrophobic side chain) residues of different composition but constant length (Doud *et al.*, 1993). A clear correlation was observed between the hydrophobicity of the core region (the proportion of Leu residues) and the efficiency of P48 cross-linking from a WT lysate (Figure 5). A minimum Leu:Ala ratio of 5:5 was found to be required for levels of P48 cross-linking comparable with that of the PhoA-WT whose signal sequence is in the same range of hydrophobicity. A similar correlation has been observed between the efficiency of translocation of these mutants *in vivo* (measured as rate of precursor processing) and the Leu:Ala ratio of their signal peptide (Doud *et al.*, 1993). Thus, it is tempting to speculate that the ability to bind the SRP is important for proper targeting and translocation of (mutant) prePhoA in *E.coli*. The strongly enhanced cross-linking of the very hydrophobic mutant signal peptides (Leu:Ala ratios of 7:3 and 9:1) to P48 is not proportional to the slight increase in transport activity these mutants display compared with PhoA-WT, indicating that they do not offer a particular advantage *in vivo*. Conceivably, these signal peptides may bind P48 too strongly which could affect later stages in the targeting cascade, for instance the release of the SRP. A similar correlation has been reported between the hydrophobicity of the core region of *Saccharomyces cerevisiae* vacuolar protein carboxypeptidase Y and its transport in mammalian cells (Bird *et al.*, 1990). Again, a minimum of five hydrophobic residues was found to be required in the core region of a functional signal peptide. This is in agreement with our observation that mammalian SRP displayed the same dependence on hydrophobicity for cross-linking to nascent PhoA as P48. These results suggest similar binding specificities of SRP54 and P48 which is not surprising in view of their evolutionary conservation (Bernstein *et al.*, 1989; Römisch *et al.*, 1989).

Like SRP54, P48 is the major component interacting with SA sequences of nascent integral membrane proteins of different transmembrane orientation (reviewed in High and Dobberstein, 1992). Analogous to the situation in eukaryotic systems, this class of membrane proteins may be particularly dependent on a functional SRP route to allow co-translational insertion into the membrane and thus to avoid the cytoplasmic exposure of strongly hydrophobic sequence domains in the nascent polypeptide. This would explain why cell division, which involves a complex machinery of inner membrane proteins (e.g. FtsQ, tested in this study), is quickly affected after depletion of P48 (Phillips and Silhavy, 1992), 4.5S RNA (Poritz *et al.*, 1990; Ribes *et al.*, 1990) or FtsY (Luirink *et al.*, 1994). It would also explain the essential nature of these components. This notion is supported by recent evidence indicating that membrane insertion of the integral inner membrane protein LacY is dependent on P48 *in vivo* (J.MacFarlane and M.Müller, personal communication).

Interactions with TF

To our surprise, in addition to P48, TF was found cross-linked to all tested nascent presecretory proteins and, in contrast to P48, to nascent non-signal sequence bearing proteins. Cross-linking was dependent on the context of the ribosome which is in agreement with the affinity of TF for ribosomes. Approximately 25% of TF is associated with the large subunit of ribosomes in *E.coli* lysate (Lill *et al.*, 1988). Apparently, TF is also able to associate with the eukaryotic ribosomes in our wheat germ translation system. The exact contribution of the nascent chain to this association remains to be established.

What would be the role of TF in protein translation and translocation? We propose that TF is a general nascent chain binding protein that functions as a chaperone very early in translation to prevent improper intra- and intermolecular interactions of the nascent polypeptide emerging on the surface of the large ribosomal subunit. Thus, it would function prior to the cascade of interactions involving molecular chaperones like DnaK, DnaJ, GrpE and GroEL/GroES that mediate folding of the polypeptide to its native structure in an ATP-dependent fashion (reviewed in Hartl and Martin, 1995). It may even direct these proteins to the nascent polypeptide or have a function in initial folding events itself. It is of interest to note that Hendrick and co-workers observed cross-linking of purified DnaJ at relatively high concentrations to nascent ffLuc of the same length and in a similar experimental set-up to that used in this study (Hendrick *et al.*, 1993). In contrast, we did not observe cross-linking to DnaJ when using WT lysate, TF-depleted lysate, lysate from a DnaJ overproducing strain or lysate prepared from heat-shocked cells (data not shown). The reason for this difference remains unknown at present.

TF was originally implicated in protein translocation due to its interaction with purified proOmpA upon the removal of urea by dialysis or dilution (Crooke and Wickner, 1987). This interaction stimulated the translocation of proOmpA into inverted inner membrane vesicles leading to the hypothesis that TF is a chaperone that maintains the translocation competent conformation of proOmpA (Crooke and Wickner, 1987; Crooke *et al.*, 1988a,b). However, cells depleted of TF did not exhibit pleiotropic secretion defects (Guthrie and Wickner, 1990). These apparently contradictory observations may be explained by a general affinity of TF for unfolded polypeptides either during translation at the ribosome or unfolded by reversible denaturation of the completed protein. The latter association may prevent degradation and aggregation, as has been shown for proOmpA (Crooke *et al.*, 1988a). Notably, eukaryotic SRP was able to replace TF and stabilize proOmpA for translocation in a similar experiment (Crooke *et al.*, 1988b; Sanz and Meyer, 1988) indicating that both TF and SRP which act co-translationally *in vivo*, may stabilize protein conformation or rather the lack of conformation *in vitro*.

Increased levels of TF were found to compete with the interaction of P48 with nascent prePhoE (Figure 9A). This might explain why overproduction of TF aggravates the export defect in a *SecB* null strain rather than relieving it (Guthrie and Wickner, 1990). TF might prevent interaction of the SRP with nascent chains in these cells thereby blocking an alternative route of protein targeting. On the other hand, depletion of TF results in increased interaction of nascent preproteins with the SRP *in vitro* (Fig 9A) which is consistent with the stimulation of proOmpA export upon TF depletion in a *SecB* null background observed *in vivo* (Guthrie and Wickner, 1990).

The binding characteristics of TF are reminiscent of

the mammalian NAC which also binds to short nascent chains in the context of the ribosome (Wiedmann *et al.*, 1994). NAC is considered to confer specificity on the subsequent interaction of SRP with signal sequences since in the absence of NAC, SRP interacts with non-signal sequence bearing proteins and promotes their targeting to the ER albeit inefficiently (Wiedmann *et al.*, 1994). No such function could be assigned to TF since interaction of SRP to non-signal sequence regions upon depletion of TF was not observed. Furthermore, purified P48 appeared to be fully capable of distinguishing between functional and non-functional mutant PhoA signal sequences (data not shown) arguing against a NAC-like role for TF.

In conclusion, we provide evidence that TF interacts with all short nascent polypeptides whereas P48 interacts specifically with nascent chains bearing a functional signal sequence or SA sequence, irrespective of whether SRP-dependent or -independent targeting pathways are used *in vivo*. This is consistent with TF having a 'global' role in protein synthesis and P48 playing a specialized role in protein targeting. Experiments are underway to investigate the interactions of *E.coli* components with longer nascent chains to get more insight into the sequence of folding and targeting events when polypeptides are being synthesized at the ribosome.

Materials and methods

Materials

Restriction enzymes and RNasin were from Boehringer Mannheim GmbH (Mannheim, Germany). The Taq DyeDeoxy Terminator Cycle Sequencing Kit was from Applied Biosystems (Foster City, CA). T7 RNA polymerase, SP6 RNA polymerase, pGEM3Z and pGEM4Z were from Promega Biotech (Madison, WI). Cycloheximide and 7-methylguanosine 5′-monophosphate were supplied by Sigma Chemical Co. (St Louis, MO). The cap analogue $m^7G(5')ppp(5')G$ and [^{35}S]methionine were from Amersham Int. (Buckinghamshire, England). 4-(3-trifluoromethyldiazirino)benzoic acid (TDBA) was purchased from Photoprobes (Knonau, Switzerland). Purified P48 and TF were gifts from I.Sinning and E.-J.Breukink respectively.

Preparation of cell lysates

Escherichia coli cell lysates were prepared as described previously (Luirink *et al.*, 1992). Strain MC4100 harboring either pDS12 or pDS12-48 was used to obtain WT lysate or P48-enriched lysate respectively (Ribes *et al.*, 1990; Luirink *et al.*, 1992). TF-enriched lysate was prepared from strain DH5α harboring pTIG2 which contains the cloned TF gene under transcriptional control of the arabinose promoter (Guthrie and Wickner, 1990). This strain was grown overnight in YT medium supplemented with 0.2% glucose, diluted 50-fold in YT medium supplemented with 0.2% glucose and 0.2% arabinose and grown for 4 h before lysates were prepared. To obtain P48-depleted or TF-depleted lysate, strains WAM113 (Phillips and Silhavy, 1992) and BG87 (Guthrie and Wickner, 1990) were used, respectively. These strains harbor the chromosomal P48 or TF gene under control of the arabinose promoter. They were grown overnight in YT medium supplemented with 0.2% glucose and 0.2% arabinose, washed three times in YT and diluted 50-fold in YT supplemented with 0.2% glucose. Cells were kept in the exponential growth phase until a filamented phenotype became apparent and lysates were prepared. Overproduction and depletion were checked by SDS–PAGE and Western blotting.

Plasmid constructs

All constructs used were cloned under transcriptional control of the phage T7 or SP6 promoter and the N-terminal regions of the encoded products are shown in Figure 2. pGEM-Luc carrying the gene for firefly luciferase was from Promega. pBSK-PhoE-WT encoding wild-type PhoE was a gift of Jan Tommassen. pBSK-PhoE-Δss was prepared by exchanging the small *Pac*I–*Mlu*I fragment from pBSK-PhoE-WT encompassing the signal sequence coding region for that of pJP370 which encodes PhoE lacking a signal sequence coding region (de Cock *et al.*, 1990). pGEM4Z-Lpp was prepared by subcloning the *Xba*I–*Eco*RI fragment from pYM141 (Morinaga *et al.*, 1984) encompassing the complete Lpp coding region into pGEM4Z. pSP65-M13coat (Gallusser and Kuhn, 1990) and pGEM1-Lep (Nilsson *et al.*, 1994) harbor the complete M13 coat and Lep coding regions respectively. pGEM3Z-FtsQ-WT was created by subcloning the PCR amplified 5′ end of the FtsQ gene using pUC18-FtsQ (a gift of Nienke Buddelmeier) as template. The left flanking primer (5′-ACG*TCTAGA*GCCGCCACCATGTCGC-AGGCTGCTCTG-3′) was extended with a 'Kozak consensus sequence' (Kozak, 1989) immediately 5′ to the ATG initiator codon (underlined) and an *Xba*I site (italics) at the 5′-end. The right flanking primer complementary to the codons 102–108 (5′-GTCGG*AAGCTT*AATCC-ACGGCAGGC-3′) was extended to include a *Hin*dIII site (italics). The PCR product was gel purified, cut with *Xba*I and *Hin*dIII and cloned between the same sites in pGEM3Z. pGEM3Z-FtsQ-msa was created by site directed mutagenesis using successive PCR essentially as described (Higuchi *et al.*, 1988) using pGEM3Z-FtsQ-WT as template and the flanking primers described above. The following mutagenic oligonucleotides were used: (5′-GACCGTTTTAAAGACAGTGTTGAAGAGCGGCTG-3′) and (5′-CAGCCGCTCTTCAACACTGTCTTTAAAACGGTC-3′) in which the mutated nucleotides are underlined. The final mutated PCR fragment was cut and cloned as described above.

pGEM3Z-PhoA-WT and its mutant derivatives were created by subcloning the PCR amplified 5′ end of the (mutant) PhoA gene using (mutant) pCASS3 (Doud *et al.*, 1993) as template. The left flanking primer (5′-TTC*GAATTC*GCCGCCACCATGAAACAGTCGACT-3′) was extended with a 'Kozak consensus sequence' (Kozak, 1989) immediately 5′ to the GTG initiator codon which was mutated into an ATG initiator codon (underlined) to allow initiation of translation in wheat germ extracts and an *Eco*RI site (italics) at the 5′-end. The right flanking primer complementary to the codons 78–84 (5′-GAG*TCTAGA*CGT-GCGGCAGTAATTTCC-3′) was extended to include an *Xba*I site (italics). The PCR product was gel purified, cut with *Eco*RI and *Xba*I and cloned between the same sites in pGEM3Z.

The nucleotide sequences of the cloned (mutant) genes were confirmed by DNA sequencing using the 373A Automated DNA Sequencer of Applied Biosystems. It should be noted that sequence analysis of pGEM3Z-PhoA-9A1Lh and its template revealed that it contains a point mutation resulting in substitution of the fourth alanine residue in the core region for a threonine residue. This substitution did not affect the predicted hydrophobicity of the signal sequence. Moreover, the mutation has been corrected by site directed mutagenesis resulting in a signal sequence that was equally defective in promoting transport *in vivo* (Doud *et al.*, 1993).

In vitro transcription, translation and photo cross-linking

For photo cross-linking we used truncations of the various presecretory and membrane proteins to allow exposure of the signal sequence or of the first trans-membrane segment just outside the ribosome. Truncation was achieved by using truncated messenger RNAs coding for the N-terminal region of the protein (PhoE-WT, PhoE-Δss, FtsQ-WT, FtsQ-msa, pGEM-luc, PhoA-WT and mutant derivatives; Figure 2) or by using full-length transcripts and inclusion of complementary oligonucleotides at 40 μg/ml in the translation system (Haeuptle and Dobberstein, 1986).

Plasmids were linearized with *Mlu*I (pBSK-PhoE-WT and pBSK-PhoE-Δss), *Eco*RI (pGEM4Z-Lpp), *Hin*dIII (pSP65-M13coat, pGEM3Z-FtsQ-WT and pGEM3Z-FtsQ-msa) *Bam*HI (pGEM1-Lep), *Xba*I (pGEM3Z-PhoA-WT and mutant derivatives) or *Hin*fI (pGEM-Luc) and transcribed as described by the manufacturer (Promega). The resulting messenger RNAs were translated in a wheat germ cell-free system as previously described (High *et al.*, 1991a). The transcripts derived from pGEM4Z-Lpp, pSP65-M13coat and pGEM1-Lep were truncated during the translation reaction using the oligonucleotides Lpp-trunc1 (5′-CACAGGTACTATTACTTGCG-3′), M13-trunc1 (5′-ATTGTATCGGTTTATCAGCT-3′) and Lep-trunc1 (5′-AAAATCACC-AATTAACAGAG-3′), respectively. In order to create PhoE-trunc1, full length transcript of pBSK-PhoE-WT was translated in the presence of the oligonucleotide PhoE-trunc1 (5′-GCAAACTCTGCTTCCCA-3′).

After cell-free translation in the presence of ε-TDBA-Lys-tRNA, the ribosome-nascent chain complexes were purified by centrifugation through a high salt/sucrose cushion as described (High *et al.*, 1991a). The products were incubated in the presence of *E.coli* lysate or purified proteins and irradiated to induce photo cross-linking (Figure 1; Luirink *et al.*, 1992).

Analysis of photo cross-linking products

Photo cross-linking products were either TCA precipitated or immunoprecipitated under denaturing conditions. Immunoprecipitation was carried out as described (Römisch *et al.*, 1990) except that the ribosome-nascent chain complexes were repurified by centrifugation through a high salt/sucrose cushion after cross-linking and prior to immunoprecipitation to remove unreacted *E.coli* material (Luirink *et al.*, 1992).

All samples were analysed on 12% or 15% SDS–polyacrylamide gels. Radiolabeled proteins were visualized by phosphorimaging using a Molecular Dynamics PhosphorImager 473 and quantified using the Imagequant quantification software from Molecular Dynamics.

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