An amphiphilic region in the cytoplasmic domain of KdpD is recognized by the signal recognition particle and targeted to the *Escherichia coli* membrane

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Summary

The sensor protein KdpD of *Escherichia coli* is composed of a large N-terminal hydrophilic region (aa 1–400), four transmembrane regions (aa 401–498) and a large hydrophilic region (aa 499–894) at the C-terminus. KdpD requires the signal recognition particle (SRP) for its targeting to the membrane. Deletions within KdpD show that the first 50 residues are required for SRP-driven membrane insertion. A fusion protein of the green fluorescent protein (GFP) with KdpD is found localized at the membrane only when SRP is present. The membrane targeting of GFP was not observed when the first 50 KdpD residues were deleted. A truncated mutant of KdpD containing only the first 25 amino acids fused to GFP lost its ability to specifically interact with SRP, whereas a specific interaction between SRP and the first 48 amino acids of KdpD fused to GFP was confirmed by pull-down experiments. Conclusively, a small amphiphilic region of 27 residues within the amino-terminal domain of KdpD (aa 22–48) is recognized by SRP and targets the protein to the membrane. This shows that membrane proteins with a large N-terminal region in the cytoplasm can be membrane-targeted early on to allow co-translational membrane insertion of their distant transmembrane regions.

Introduction

The KdpD protein of *Escherichia coli* is a membrane component of the KdpD/E sensory system involved in maintaining the intracellular osmolarity. In cases of low K⁺ concentrations in the medium, the expression of the high-affinity K⁺ pump KdpFABC is induced (Epstein and Davis, 1970). In this signalling cascade, the C-terminal domain of KdpD is phosphorylated most likely at His673 (Voelkner et al., 1993). Subsequently, the phosphoryl group is transferred to Asp52 of the cytoplasmic response regulator KdpE, which in turn acts as a transcriptional activator for kdpFABC (Sugiura et al., 1992; Nakashima et al., 1993). Although the exact mechanism of the K⁺ sensing is still unknown, the C-terminal domain of KdpD is by itself functional (Rothenbücher et al., 2006).

The biosynthesis of KdpD has been studied in detail. The KdpD protein consists of a large cytoplasmic N-terminal domain, four closely spaced transmembrane regions, and an extended cytoplasmic C-terminal domain (Fig. 1). The insertion of the transmembrane regions occurs independently of SecA, SecE and YidC, but involves the electrochemical membrane potential (Facey and Kuhn, 2003). As the translocated periplasmic region P1 between the first and second transmembranes is only four residues long, it was extended to 19 residues with an antigenic epitope. The mutant showed the same insertion characteristics for the translocation of the P1 region as the wild-type protein and was Sec- and YidC-independent. It was therefore concluded that short periplasmic loops can translocate without assistance of other known proteins (Facey and Kuhn, 2003).

In *E. coli*, there are multiple pathways for directing proteins to and across the inner membrane. The twin-arginine translocation system (Tat) targets a small group of cofactor-containing proteins across the cytoplasmic membrane via distinct signal peptides bearing a conserved RR motif (Alami et al., 2003). The Tat pathway transports only folded proteins post-translationally through the Tat translocon. The majority of secreted *E. coli* proteins are synthesized as preproteins with a cleavable signal peptide at their N-terminus. These preproteins are targeted to the cytoplasmic membrane post-translationally by the molecular chaperone SecB via the cytosolic ATPase SecA which is associated with the SecYEG translocon (Driessen et al., 2001). The core of the Sec translocon consists of the integral membrane
proteins SecY, SecE and SecG, and the peripheral subunit SecA. The Sec translocase mediates the stepwise translocation of secretory proteins across the membrane. In contrast, most cytoplasmic membrane proteins do not contain a cleavable signal peptide and their N-terminal transmembrane segment serves as the membrane targeting signal. Most inner membrane proteins are targeted in a co-translational manner to the SecYEG translocase by the signal recognition particle (SRP) and its receptor (Herskovits et al., 2000).

The bacterial SRP consists of a 48 kDa protein Ffh (Fifty-four homologue) and a 4.5S RNA (Keenan et al., 2001; Luirink and Sinning, 2004). Together with its receptor FtsY, SRP delivers membrane and secretory proteins to the translocation channel in the plasma membrane. In E. coli, Ffh, FtsY and the 4.5S RNA are all essential for viability. Ffh consists of three domains: the N-terminal N domain (α-helical domain), the G domain (nucleotide binding domain) and the C-terminal M domain (methionine-rich, α-helical domain). SRP-binding substrates are bound by the methionine-rich M-domain of Ffh when they leave the ribosomal exit channel. There, a hydrophobic ‘finger loop’ of Ffh is proposed to interact with an α-helical substrate region, such as an uncleaved signal sequence (Luirink and Sinning, 2004). In E. coli, usually the first transmembrane region of multi-spanning membrane proteins interacts with SRP (Valent et al., 1997; Beck et al., 2000). The substrate specificity of SRP for membrane proteins may reflect the higher affinity of SRP for hydrophobic signal sequences. In vitro cross-linking studies have shown that the efficiency of cross-linking to Ffh is correlated with the hydrophobicity of the signal sequence (Valent et al., 1997; 1998). Furthermore, E. coli presecretory proteins can be re-routed into the SRP pathway by increasing the hydrophobicity of their signal sequences (Lee and Bernstein, 2001).

In the SRP-mediated targeting pathway, SRP binds to the hydrophobic signal sequence of the nascent chain as it emerges from the translating ribosome. The resulting SRP–ribosome nascent chain complex is then targeted to FtsY at the membrane where an interaction involving GTP between SRP and its receptor catalyses the dissociation of the nascent chain from SRP. The nascent membrane proteins are then inserted into the SecYEG complex (for a review see Keenan et al., 2001).

In the present study, we investigated in detail the initial interactions of the inner membrane protein KdpD with SRP. The membrane insertion of KdpD depends on SRP as we demonstrate with an Ffh- and an FtsY-depletion strain. We also show that a short sequence in the N-terminal cytoplasmic domain of the KdpD protein binds to SRP. A fusion protein of the green fluorescent protein (GFP) with KdpD is targeted to the membrane when the SRP-binding motif is present, but remains in the cytoplasm when this element is removed.

Results

Signal recognition particle is required for the membrane insertion of KdpD

The sensor kinase protein KdpD inserts into the membrane independently of the Sec translocase and YidC (Facey and Kuhn, 2003). To investigate the involvement of SRP in the biogenesis of KdpD, we transformed the Ffh-depletion strain WAM121 with the plasmid (pSF51) encoding the wild-type KdpD. In this strain, the ffh gene expression is under the control of the arabinose-inducible araBAD promoter (de Gier et al., 1997; 1998). Ffh is depleted when the cells are grown in the presence of glucose and absence of arabinose. WAM121 cells were grown in the presence of glucose or arabinose, respectively. The cells were induced, labelled with [35S]methionine for 1 min, chased for 2 min, immediately converted to spheroplasts and treated with proteinase K. In a recent study, we observed that proteinase K did not cleave the protein in the first periplasmic loop P1, probably because of the short size of the loop (four amino acid residues). Cleavage in the second periplasmic loop P2 (10 amino acid residues) occurred only partially and led to a protease-protected fragment of 47 kDa which was recognized by the KdpD antibody (Facey and Kuhn, 2003). The results show that when Ffh was present, the translocation of the second periplasmic loop of KdpD was followed by the generation of the C-terminal 47 kDa proteolytic fragment (Fig. 2A, lane 2). In contrast, in Ffh-depleted cells, the formation of the C-terminal fragment was affected (compare lanes 2 and 5). Depletion of Ffh reduced the efficiency of the translocation of the second periplasmic loop of KdpD as indicated by the reduced appearance of the C-terminal fragment. This suggests that SRP is required for membrane insertion of KdpD. Following lysis of the cells with detergent, we confirmed that the
smaller fragment was readily digested (lanes 3 and 6). As a control, the cytoplasmic protein GroEL and the outer membrane protein OmpA were analysed in parallel. The OmpA antibody used in this study recognizes the periplasmic domain of OmpA and the degradation of OmpA indicates that the protease is active in the periplasm. Depletion of the Ffh component of SRP largely inhibited KdpD insertion, without affecting the Sec-dependent export of OmpA. Depletion of Ffh was confirmed by analysing the Ffh content in a cell sample taken before induction with IPTG by immunoblotting using Ffh antiserum (Fig. 2B).

The membrane insertion of KdpD was further investigated in the FtsY-depletion strain IY26. Cells of strain IY26 in which ftsY expression is under control of an arabinose-inducible promoter were grown in the presence (FtsY⁺, Fig. 2C, lanes 1–3) or absence of arabinose (FtsY⁻, Fig. 2C, lanes 4–6) and then assayed for translocation. In accordance with the effects of depletion of Ffh, depletion of FtsY also inhibits membrane insertion of KdpD (compare lanes 2 and 5). Together, the in vivo results show that KdpD requires SRP for efficient membrane insertion. Furthermore, the processing of OmpA was unaltered when Ffh or FtsY were depleted, indicating that the Sec machinery was still functional. Depletion of FtsY was verified by analysing the FtsY level in a cell sample in parallel by immunoblotting with FtsY antiserum (Fig. 2D).
To test whether GFP fused to KdpD is also targeted by SRP, we analysed the translocation of a truncated KdpD fragment fused after amino acid residue 448 to GFP in the Ffh-depletion strain WAM121. The truncated fragment termed KdpD-N (i.e. coding the amino acids 1–448 of KdpD) has been described earlier (Facey and Kuhn, 2003). To analyse the translocation of this fragment, a short HA-epitope was introduced between helices 1 and 2 (Facey and Kuhn, 2003). It was previously shown that the first periplasmic loop of KdpD with an HA-epitope tag inserts into the inner membrane independent of YidC and the Sec components like wild-type KdpD (Facey and Kuhn, 2003). We investigated the effect of Ffh depletion on the translocation of KdpD-N fused to GFP in the strain WAM121. Here, the epitope-tagged KdpD-N(HA)–GFP fusion protein was immunoprecipitated with antiserum to HA. As described above, when Ffh is present (cells grown with arabinose), KdpD-N(HA)–GFP was readily inserted into the membrane and the exposed HA tag was digested with proteinase K (Fig. 3A, lanes 1 and 2). In Ffh-deficient cells (grown with glucose), the insertion of KdpD-N(HA)–GFP was inhibited. The protein was not accessible to the externally added proteinase K, indicating that it remains in the cytoplasm (Fig. 3A, compare lanes 4 and 5). These results are in agreement with the observations from the wild-type KdpD that SRP is required for membrane insertion. The fused GFP protein has no influence on the membrane targeting and insertion of KdpD. Interestingly, we show here that alone the N-terminal domain of KdpD requires SRP. Depletion of Ffh was confirmed by analysing the Ffh level in a cell sample in parallel by immunoblotting with Ffh antiserum (Fig. 3B).

The KdpD–GFP fusion protein is localized at the bacterial membrane

In order to visualize the localization of KdpD, we fused GFP to wild-type KdpD. To study the involvement of SRP in KdpD–GFP membrane targeting, we analysed the localization of KdpD–GFP in the Ffh-depletion strain WAM121 and FtsY-depletion strain IY26. When the cells expressing only GFP under arabinose conditions were examined by fluorescence microscopy, the fluorescence was uniformly distributed both in the WAM121 (Fig 4A) and in the IY26 cells (Fig. 4B). Cells bearing the plasmid pJFBD/GFP coding for KdpD–GFP were grown under arabinose conditions (Ffh+/FtsY+) and glucose (Ffh−/FtsY−) and examined by fluorescence microscopy, the fluorescence was found primarily located at the inner membrane (Fig 4C and E). However, when the cells were grown in the presence of glucose to deplete Ffh or FtsY (Fig 4D and F), the fusion protein was found uniformly located throughout the cytoplasm. These results are in agreement with the protease accessibility assay with wild-type KdpD that the SRP component Ffh and the SRP receptor FtsY are required for efficient targeting of KdpD to the membrane.

Deletions in the N-terminal domain of KdpD affect the topology

To gain insight into the signal sequence required for targeting of KdpD to the inner membrane with SRP, genetic variants were constructed with truncations in the N-terminal domain of KdpD–GFP. Because SRP acts at a very early stage in protein biosynthesis, one would expect the signal sequence to be at the beginning of a nascent chain because of co-translation of the protein binding to SRP. In most bacterial inner membrane proteins, the N-terminal transmembrane segment serves as the membrane targeting signal. However, the first transmembrane segment of KdpD is 400 amino acid residues from the N-terminus of KdpD. MC1061 cells were transformed with the plasmids coding for wild-type KdpD–GFP, KdpDΔ50–GFP, KdpD-N–GFP, KdpD-NΔ50–GFP and GFP (Fig. 5) and examined by fluorescence microscopy. The results show that when the first 50 N-terminal amino acid residues were deleted from KdpD–GFP, the fusion protein (KdpDΔ50–GFP) was distributed uniformly throughout the cytoplasm (Fig. 5C) like GFP alone (Fig. 5D). In contrast, more than 87% of total fluorescence of KdpD–GFP was located at the inner membrane (Fig. 5A). Similarly, KdpD-N–GFP composed of the cyto-
plasmic N-terminal region and the first two transmembrane regions of KdpD showed more than 99% of total fluorescence located at the membrane (Fig. 5B). In contrast, when the first 50 N-terminal amino acid residues were deleted from KdpD-N–GFP, the fusion protein (KdpD-N\(_{50}\)–GFP) was distributed uniformly throughout the cytoplasm (Fig. 5E).

The targeting of the N-terminal fragment of KdpD lacking the first 50 amino acid residues was investigated in the Ffh-depletion strain WAM121. To analyse the translocation of this fragment, a short HA-epitope was introduced between helices 1 and 2. Cells expressing the mutant protein (KdpD-N\(_{50}\)(HA)–GFP) were grown in medium supplemented with arabinose (Fig. 6). When Ffh was present, proteolysis of the N-terminal fragment of KdpD lacking the first 50 amino acid residues was inhibited (lane 2). Deletion of the first 50 amino acid residues of KdpD impaired the insertion of KdpD into the membrane. These results suggest that within the first 50 amino acid residues of KdpD, a sequence is present which targets KdpD to the inner membrane with SRP.

A 48-residue-long peptide fused to GFP leads to its membrane localization but not its insertion

To further characterize the SRP binding sequence for KdpD, additional kdpD–gfp gene fusions were constructed by fusing the first 25 and 48 N-terminal amino acid residues of KdpD to GFP to construct N25–GFP and N48–GFP, respectively. Synthesis of the GFP fusion proteins was induced either with IPTG for KdpD–GFP or arabinose for N25–GFP and N48–GFP for 1 h. At this point, the transcription inhibitor rifampicin was added to prevent further synthesis of the proteins. The localization of the fusion proteins was observed by fluorescence microscopy after 5 min (Fig. 7A–C), 30 min (Fig. 7D–F)
and 2 h post rifampicin addition (Fig. 7G–I). Similar to the results seen with the full-length KdpD–GFP hybrid, transformants expressing N48–GFP (residues 1–48 of KdpD fused to GFP) showed a halo of fluorescence at the membrane in most of the cells at about 5 and 30 min after the addition of rifampicin (Fig. 7A and D). However, 2 h later, almost all the cells expressing N48–GFP showed the fusion protein uniformly distributed throughout the cytoplasm (Fig. 7G). These results imply that for N48–GFP, first the halo formation occurs in which N48–GFP is targeted to the inner membrane by the SRP pathway. Within the next 90 min, the protein is distributed back to the cytoplasm (Fig. 7G), most likely because no hydrophobic transmembrane region is present in the fusion protein, and therefore no insertion into the membrane can take place. We attempted to study the kinetics of membrane localization by following individual cells, but this was unsuccessful because the fluorescence was bleached after a few excitations. In contrast to N48–GFP, in cells

**Fig. 5.** Deletion of the first 50 amino acids of KdpD inhibits its targeting to the membrane. *E. coli* MC1061 expressing KdpD–GFP (A), KdpD-N–GFP (B), KdpDΔ50–GFP (C), GFP (D) and KdpD-NΔ50–GFP (E) were examined by fluorescence microscopy. After induction, transcription was blocked by the addition of rifampicin (1 mg ml⁻¹, final concentration) for 45 min. The bar represents 5 μm.

**Fig. 6.** Deletion of the first 50 amino acid residues of KdpD impaired the insertion of KdpD into the membrane. *E. coli* WAM121 cells were transformed with the plasmid (pMS-NΔ50/GFP) containing the HA epitope-tagged KdpD-N protein (i.e. coding the amino acid residues 51–448 of KdpD) lacking the first 50 amino acid residues of KdpD fused to GFP. The cells were grown in the presence of arabinose as described in Fig. 2A. Cells were induced with 1 mM IPTG for 10 min, pulse-labelled with [³⁵S]methionine for 1 min and chased with non-radioactive methionine for 2 min. The cells were then converted to spheroplasts and treated with or without proteinase K for 1 h. The epitope-tagged KdpD-N protein was immunoprecipitated with antisera to HA (for the epitope in the first periplasmic loop), GroEL and OmpA, and then analysed by SDS-PAGE and visualized by phosphorimaging.
expressing KdpD–GFP (Figs 7B, E and H) the membrane localization was kept at least for 2 h. Both proteins, N48–GFP and KdpD–GFP were not proteolytically digested during the 2 h as verified by Western blotting (data not shown). Cells expressing N25–GFP encoding the first 25 N-terminal amino acid residues of KdpD with GFP were found uniformly located throughout the cytoplasm at all expression times (Figs 7C, F and I).

A control is included to show what happens to the GFP fusion proteins in the absence of rifampicin addition without inhibiting the transcription and therefore protein synthesis (Fig. 7J–L). N48–GFP showed not only a halo of fluorescence at the membrane, but also the fusion protein uniformly distributed throughout the cytoplasm in the absence of rifampicin after 2 h (Fig. 7J). Cells expressing KdpD–GFP in the absence of rifampicin (Fig. 7K) showed the protein mainly located at the membrane like in the presence of rifampicin (Fig. 7H). When the N25–GFP fusion protein was examined by fluorescence microscopy without rifampicin (Fig. 7L), the fusion protein was found uniformly located throughout the cytoplasm like in the presence of rifampicin (Figs 7C, F and I).

To exclude the possibility that the cytoplasmic localization of the GFP fusion proteins during the 2 h of rifampicin

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Fig. 8. Western blot analysis of Ffh and FtsY levels in cells expressing the fusion proteins in the presence or absence of rifampicin. After induction for 1 h, cells were either treated with rifampicin (1 mg ml⁻¹, final concentration) or without rifampicin for 5, 30 and 120 min as described in Fig. 7. Immunoblotting was done using antiserum against Ffh (upper panel) and FtsY (lower panel), respectively.

The 48-residue peptide of KdpD binds to SRP

To test whether the N-terminal signal element is capable of directly binding to SRP in vitro, we investigated the interaction between N48–GFP (residues 1–48 of KdpD fused to GFP) and N25–GFP (residues 1–25 of KdpD fused to GFP) with the His-SRP protein by pull-down assays. To carry out these experiments, the GFP fusion proteins were expressed in E. coli MC1061 and purified by immobilized metal affinity chromatography (Ni-IMAC). GFP contains 10 histidine residues which interact with Ni(II) ions (Li et al., 2001). To avoid GFP also binding to the Ni Sepharose column for the pull-down assays, the GFP fusion proteins were added to the column in equilibration buffer containing 25 mM imidazole. In the assay, the His-SRP protein was attached to the Ni Sepharose column (Fig. 11, lane 1L) and then incubated with one of the fusion proteins (Fig. 9). As expected, the fusion protein is not integrated into the membrane because no hydrophobic transmembrane segment is present. Our results indicate that the region of the first 48 amino acid residues of KdpD is required for SRP-dependent targeting, whereas the transmembrane regions of KdpD are required for insertion into the membrane.

We then generated a smaller construct containing the amino acid residues 22–48 of KdpD fused to GFP. This plasmid coding for N22–48/GFP was transformed in E. coli MC1061. Synthesis of the N22–48/GFP fusion protein was induced with arabinose and cells were grown for 1 h. At this point, the transcription inhibitor rifampicin was added to prevent further synthesis of the GFP fusion proteins and the localization of N22–48/GFP was observed by fluorescence microscopy after either 5 min (Fig. 10A), 30 min (Fig. 10B) or 2 h (Fig. 10C). When the N22–48/GFP fusion protein was examined by fluorescence microscopy after 5 and 30 min, the fusion protein was primarily located at the inner membrane (Fig. 10A and B). It appears that like N48–GFP, newly synthesized N22–48/GFP is first targeted to the membrane and then transferred back to the cytoplasm (Fig. 10C). The absence of a transmembrane region inhibits insertion of the fusion protein into the membrane. This result is consistent with the notion that SRP is required for membrane targeting and subsequently the transmembrane regions of KdpD for insertion into the membrane. N22–48/GFP was not proteolytically digested during the 2 h as verified by Western blotting (data not shown). Without rifampicin addition, cells expressing N22–48/GFP showed the fusion protein not only located at the membrane but also uniformly distributed throughout the cytoplasm (Fig. 10D), like for N48–GFP (Fig. 7J).

Fig. 9. The first 48 amino acids of KdpD do not translocate GFP across the membrane. Cells of strain WAM121 (pSF165) were grown overnight in LB medium supplemented with arabinose. The overnight culture was diluted 1:20 into fresh LB medium supplemented with arabinose and grown to an OD₆₀₀ of 0.4. The cells were then transferred to M9 minimal medium for 30 min. After induction with IPTG for 10 min, the cells were pulse-labelled with [³⁵S]methionine for 1 min and chased with non-radioactive methionine for 2 min. Samples were prepared and processed as described for Fig. 2A. The N48–GFP protein was immunoprecipitated with antiserum against the N-terminus of KdpD.
the purified GFP fusion proteins (Fig. 11, lane 2L), and after extensive washing (Fig. 11, lanes 1–3), the bound proteins were eluted (Fig. 11, lanes E1–3) and analysed by SDS-PAGE and Western analysis. As shown in Fig. 11A, we found that the N48–GFP protein co-elutes from the resin with the His-tagged SRP protein (lane E1), demonstrating that the first 48 residues of KdpD physically interact with SRP. In contrast, the N25–GFP protein was unable to bind and co-elute from the resin with the His-tagged SRP protein (Fig. 11B, lane E1). As a control and evidence of the specificity of this interaction, GFP did not bind to the His-SRP protein (Fig. 11C).

As negative controls, GFP, N25–GFP and N48–GFP were added to the column without His-SRP. None of the proteins were able to bind specifically to the Ni Sepharose column under the assay conditions we used (data not shown).

These results show that the amphiphilic region including residues 22–48 of KdpD interacts directly with SRP.

Discussion

The membrane targeting of most newly synthesized integral proteins is mediated by SRP. As in E. coli nearly all membrane proteins have an N-terminal uncleavable signal peptide, the region that interacts with SRP is most likely the first transmembrane region. This ensures that early after the onset of translation, the nascent protein interacts with SRP and targets the protein co-translationally to the membrane (for a review see Luirink and Sinning, 2004). In the sensor protein KdpD, the first transmembrane region starts 401 residues after the initiating methionine preventing an early membrane targeting of the protein. Therefore, an additional signal element might be present at the N-terminus to allow co-translational membrane targeting. Our results show that the residues 22–48 of KdpD contain a signal element that is capable to bind to SRP and to target the GFP protein to the membrane.

GFP and its mutants have become an invaluable marker for monitoring protein localization and gene expression in vivo (for a review see Margolin, 2000). Using GFP fused to KdpD, we examined where the targeting information of KdpD to the inner membrane is localized. When the 400-residue-long hydrophilic domain with helices 1 and 2 (KdpD-N) was fused to GFP, we found that the fusion protein was found at the membrane (Fig. 5B). This suggested that the information for membrane targeting might lie within the first 448 residues of KdpD. Moreover, a deletion of the first 50 amino acid residues of KdpD impaired the insertion of KdpD (Fig. 5C) and KdpD-N (Fig. 5E) into the membrane. We then fused the first 48 amino acid residues of KdpD to GFP and found that this protein was readily targeted to the membrane.

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Taken together, these results show that an N-terminal signal element is required for the targeting of KdpD to the inner membrane. Although the most direct explanation of these data is that SRP targets KdpD to the membrane, it is possible that a protein targeted to the membrane by SRP is required for KdpD targeting. The results provided here clearly show that SRP (Ffh) and the SRP receptor (FtsY) are involved in the membrane targeting of KdpD. E. coli strains that can be manipulated to deplete SRP or its receptor show that KdpD insertion is impaired in the absence of Ffh (Fig. 2A) or FtsY (Fig. 2C). Also, the KdpD deletion mutant lacking the first 50 amino acid residues was incapable of inserting into the membrane as judged by the protease mapping technique (Fig. 6). These observations suggested that the N-terminal signal element of KdpD directly binds to SRP. Indeed, His-SRP was binding to the purified fusion protein N48–GFP that contained the signal element (Fig. 11A). The minimal peptide that supported the binding was N22–48 fused to GFP. This peptide (Fig. 12) contains five positively charged residues, in which three are closely spaced (aa 22–26) and a stretch of 10 hydrophobic residues (aa 27–36) followed by another six hydrophobic residues (aa 38–43). In general, a highly hydrophobic signal anchor region is the binding element for SRP-mediated co-translational membrane insertion of integral membrane proteins (de Gier et al., 1998; Peterson et al., 2003). In addition, basic amino acids promote binding of the signal peptide with SRP through electrostatic interactions (Peterson et al., 2003). The crystal structure of a complex between the E. coli Ffh M domain and a fragment of the 4.5S RNA has revealed an unusual RNA–protein interface that is thought to constitute the signal peptide binding groove (Batey et al., 2000). It has been suggested that this binding site composed of both protein and RNA would accommodate a signal peptide through a combination of hydrophobic interactions and electrostatic contacts. Therefore, the three closely spaced basic residues (Arg and Lys) in the peptide might enhance the SRP binding and compensate the lower hydrophobicity of the KdpD signal element. It would be interesting to exchange the lysyl and arginyl residues in the peptide into neutral amino acid residues to see if the peptide still interacts with SRP. Within this peptide, a Walker A motif was found from residues 30–38 (Jung and Altendorf, 1998). This motif is very similar to the classical ATP binding site of many ATP-requiring enzymes (Walker et al., 1982). It is conceivable that shortly after its exit from the ribosome, the SRP binds to the motif and delivers the protein to the membrane where it is released. The KdpD protein is then folded and the Walker A motif is subsequently used for an

Fig. 11. Interaction of the amino acid residues 1–48 of KdpD with His-SRP. Pull-down assays of N48–GFP (A), N25–GFP (B) and GFP (C) with His-SRP. About 5 μg of His-SRP protein (Ffh + 4.5S RNA) was attached to a Ni Sepharose column (lane 1L) which had been pre-equilibrated with 1.5 ml of equilibration buffer as indicated in Experimental procedures. Unbound protein was collected by centrifugation (lane 1F). Purified GFP and GFP fusion proteins (~5 μg) were incubated with immobilized His-SRP for 1 h at 4°C (lane 2L). Again unbound protein was collected by centrifugation (lane 2F). After three washes (lanes 1–3), bound proteins were eluted with 500 mM imidazole (lanes E1–3) and separated by SDS-PAGE. Gels were silver-stained (upper panels). Bound proteins were identified by Western blotting with anti-Ffh (middle panels) and anti-GFP (lower panels). L, load; F, flow-through; E, elution.

Fig. 12. The amino acid sequence of the N-terminal signal element that is required for targeting of KdpD to the inner membrane. The minimal peptide of 22–48 is underlined. Hydrophobic amino acids are shaded dark. Marked with a box resembles the sequence of a Walker A motif.
ATP-binding site. The N-terminal KdpD peptide is the first case that a non-transmembrane sequence provides binding to SRP and is capable to target the hydrophilic GFP to the membrane surface. As the SRP-signal peptide from KdpD is soluble, it provides an attractive tool for future SRP binding studies. The Ffh and FtsY dependence of KdpD is a further indication that the N-terminal peptide is functional in membrane targeting.

Analysis of the first 48 amino acid residues of KdpD fused to GFP indicates accumulation mainly at the inner membrane but not insertion into the membrane (Fig 7A and D, Fig 9). We assume that after contacting FtsY, GTP is rapidly hydrolysed and the GFP fusion protein is released from SRP. With no transmembrane regions for anchoring into the membrane, the protein slowly distributes throughout the cytoplasm after some time (Fig. 7G). It appears that the cells show a punctuate fluorescence staining which may reflect the formation of protein clusters at the cell periphery (Figs 4C, Fig 10A and B). The N-terminal KdpD peptide is required for SRP-dependent targeting, whereas the transmembrane regions of KdpD are required for insertion into the membrane. The data here are consistent with a co-translational targeting mechanism of KdpD in vivo. Usually, SRP binds to signal sequences of nascent polypeptides as they emerge from the ribosome. It has been proposed that the nature of a nascent polypeptide is already sensed in the ribosome, which may influence the binding of SRP near the nascent chain exit site (Gu et al., 2003; Ullers et al., 2003). Previous studies have shown that the E. coli inner membrane protein, Leader peptidase (Lep), is targeted cotranslationally by the SRP to the Sec-YidC insertion site in the membrane (de Gier et al., 1996; 1998; Samuelson et al., 2000). Photo cross-linking studies have shown that nascent Lep with a length of 40 amino acids and longer could be immunoprecipitated using anti-Ffh (Houben et al., 2002; 2005). Data suggest that targeting of Lep with SRP to the Sec-YidC insertion site starts at an early stage during biosynthesis of Lep when the nascent chain is 40 amino acids long. This would be in agreement with our results that SRP binds remarkably early during biogenesis of KdpD.

As most of the integral membrane proteins use the SecYEG translocase pathway, it was first assumed that the targeting pathways mediated by SRP converge at the translocase (Valent et al., 1998). Indeed, experimental evidence exists that show an interaction between SRP and SecYEG (Weiche et al., 2008). Cross-linking studies have demonstrated for a number of proteins a consecutive binding to SRP and Sec (Valent et al., 1998; Beck et al., 2000). More recently, it was shown that the Sec-independent protein MscL is targeted by SRP, but uses YidC for membrane insertion (Facey et al., 2007). Another example is KdpD, which translocates its two extremely short periplasmic loops in the absence of the Sec translocase and YidC (Facey and Kuhn, 2003). This underlines that targeting and insertion/translocation are functioning as separate modules and cooperate depending on the actual substrate protein.

**Experimental procedures**

**Bacterial strains and culture conditions**

Microscopy experiments were performed with *E. coli* MC1061 [hsdR mcrB araD139 (araABC-leu)7697 lacY1 galU galK rpsL thi] (Meissner et al., 1987).

For Ffh depletion, cells of *E. coli* strain WAM121 (de Gier et al., 1998) were grown overnight in LB medium containing 0.2% (w/v) arabinose, washed in medium lacking arabinose and back-diluted 1:20 in LB medium containing 0.4% (w/v) glucose. When cultures reached an OD600 of 0.4, the cells were transferred to M9 minimal medium for 30 min prior to pulse-chase labelling.

The FtsY-depletion strain IY26 (BW25113-Kan-AraC-PftsY) was obtained from I. Yosef and E. Bibi. FtsY is under the control of the araBAD promoter and operator. The FtsY-depletion strain IY26 was grown in LB medium supplemented with 0.2% (w/v) arabinose. For FtsY depletion, overnight grown cultures were first washed twice with LB medium and then back-diluted 1:40 and were grown to an OD600 of 0.4 (~4 h) in LB medium with 0.2% (w/v) glucose. Medium was switched to M9 minimal medium containing glucose and the cells were grown for an additional 30 min before labelling.

Media preparation and bacterial manipulations were performed according to standard methods (Maniatis et al., 1982). Where appropriate, ampicillin (100 µg ml⁻¹, final concentration), kanamycin (30 µg ml⁻¹, final concentration) or chloramphenicol (25 µg ml⁻¹, final concentration) were added to the medium.

**Construction of GFP fusion proteins**

The plasmids used in this study are described in Table 1. K. Jung and K. Altendorf kindly provided the plasmid pBD carrying the *kdpD* gene in pBAD18 (Jung and Altendorf, 1998). A *kdpD–gfp* fusion gene where the last codon of *kdpD* was fused to codon 1 of *gfp*mut3.1 (Cormack et al., 1996) was constructed as follows. Plasmid pJDT1 (kindly provided by S. Thompson and C. Robinson) is a pBAD24 derivative carrying a torA–gfp fusion (Thomas et al., 2001). A unique Sphl site was introduced by site-directed mutagenesis at the end of the *torA* gene. A 1.4 kb DNA fragment containing the *kdpD–C* gene (i.e. encoding residues of 444–894 of KdpD) lacking the translation stop codon was obtained by PCR and cloned as an MluI-Sphl fragment into the MluI-Sphl-digested plasmid pJDT1/Sphl to construct p24C/GFP (the *torA* gene was replaced with the *kdpD–C* gene). Plasmid p24C/GFP containing the *kdpD–C–gfp* chimeric gene was cleaved with Stul/ HindIII and inserted in Stul-HindIII-digested pBD to generate pBD/GFP encoding KdpD and GFP.

To generate the plasmid pJFBD/GFP, the coding region of KdpD–GFP was excised by SmaI-HindIII from pBD/GFP encoding KdpD and GFP.
cloned into the corresponding sites of the plasmid pSF119EH. Plasmid pBD-N50/GFP was constructed in several steps. PCR amplification with the primer set NcoI-50-KdpD and with pBD as the template produced a 1.6 kb NcoI-StuI fragment. This fragment was subsequently inserted into the NcoI-StuI site of p24C (i.e. encoding residues of KdpD, respectively. The deletion derivatives were made by digesting p33N/GFP with PstI and ligating the appropriate gel-purified fragments creating p33N–GFP. The plasmid pSF18 encoding KdpD-N (i.e. encoding residues 1–448 of KdpD with the HA-epitope) has been described (Facey and Kuhn, 2003). In this plasmid, the stop codon (TAG) was changed to GGG (glycine). The resulting plasmid pSF17-KdpD was used to construct p33N–GFP encoding KdpD-N(Gly) cleaved with XbaI-PstI and inserted into the corresponding site of pT7-7. Construction of pSH-N/GFP was done as follows: gfpmut3.1 was amplified by PCR from pGFPmut3.1 and cloned into the corresponding sites of pJF119EH. Plasmid pBD containing KdpD was cleaved with NdeI and PstI and cloned into the corresponding sites of pBAD33, to generate pSH-N/GFP encoding KdpD-N–GFP. A 2.1 kb PstI-HindIII fragment from pBAD33 was used to amplify a 1.25 kb fragment from the plasmid pSF18 encoding KdpD-N (Facey and Kuhn, 2003). This fragment was digested with NdeI and PstI and cloned into the corresponding sites of pBAD33 and ligated to MluI-SphI-digested pBD/GFP to create pSH-N/GFP. The pGFPmut3.1 gene was isolated and subsequently ligated to MluI-SphI-digested pBD/GFP to create pSh-N–GFP. Construction of pSH-N–GFP was done as follows: gfpmut3.1 was amplified by PCR from pGFPmut3.1 (Clontech) with the primers GFP-PstI and GFP-HindIII and cloned into the corresponding sites of pT7-7, to generate the plasmid pT7–GFP. From pSF51. Plasmids pBAD-N25/GFP and pBAD-N48/GFP were constructed from p33N/GFP encoding KdpD-N(Gly) cleaved with XbaI-PstI and inserted into the SmaI-HindIII-digested pMS119EH to generate pBAD-N25–GFP and pBAD-N48–GFP. The resulting plasmid pBAD-N25/GFP encoding KdpD-N(Gly) was cleaved with XbaI-PstI and inserted into the corresponding site of pBAD33 to create p33/GFP. The plasmid pBAD24 encoding KdpD was cloned into the corresponding sites of pSF165, the coding region of KdpD (i.e. encoding residues 22–48 of KdpD) was excised by XbaI-HindIII from pBAD-N25–GFP and pBAD-N48–GFP and inserted into the corresponding sites of pBAD18/GFP. The coding regions of all constructs were verified by sequence analysis.

| Plasmids | Name | Characteristics | Reference or source |
|----------|------|-----------------|---------------------|
| pBAD33   |      |                 | Guzman et al. (1995) |
| pMS119EH | pBAD33, kdpD |               | Jung and Altendorf (1998) |
| pJF119EH | pBAD33, kdpD |               | Thomas et al. (2001) |
| pSF18    | pBAD33, kdpD |               | Tabor and Richardson (1985) |
| pSF51    | pBAD33, kdpD |               | This study |
| pBD/GFP  | pBAD33, kdpD |               | This study |
| pJFBD/GFP| pBAD33, kdpD |               | This study |
| pBDN50/GFP| pBAD33, kdpD |               | This study |
| p33/GFP  | pBAD33, kdpD |               | This study |
| p33N/GFP | pBAD33, kdpD |               | This study |
| p24C     | pBAD33, kdpD |               | This study |
| p24C/GFP | pBAD33, kdpD |               | This study |
| pSH-N/GFP| pBAD33, kdpD |               | This study |
| pMS-N/GFP| pBAD33, kdpD |               | This study |
| pT7-N50(FA) | pBAD33, kdpD |               | This study |
| pSH-N50/GFP| pBAD33, kdpD |               | This study |
| pMS-N50/GFP| pBAD33, kdpD |               | This study |
| pBAD-N25/GFP| pBAD33, kdpD |               | This study |
| pBAD-N48/GFP| pBAD33, kdpD |               | This study |
| pBAD-N22–48/GFP| pBAD33, kdpD |               | This study |
| pSF163   | pBAD33, kdpD |               | This study |
| pSF165   | pBAD33, kdpD |               | This study |

CAGCTATGAAGGCGCCAGGTCCATAAATA-3' were used to amplify a 1.25 kb fragment from the plasmid pSF18 encoding KdpD-N (Facey and Kuhn, 2003). This fragment was digested with NdeI and PstI and cloned into the corresponding sites of pT7-7, to generate the plasmid pT7-N50(FA). By means of site-directed mutagenesis, the stop codon (TAG) was changed to GGG (glycine). The resulting plasmid pT7-7-N50(FA)/Gly was cleaved with XbaI-PstI and inserted into XbaI-PstI-digested p33N/GFP to create pSH-N50/GFP encoding KdpD-N50(FA)–GFP. To construct pMS-N50/GFP, plasmid pSH-N50/GFP was cleaved with XbaI-HindIII and cloned into the corresponding sites of pSF119EH. Plasmid pBD containing KdpD was cleaved with SmaI-HindIII and the fragment coding for the KdpD gene was inserted into SmaI-HindIII-digested pMS119EH to generate pSF51. Plasmids pBAD-N25/GFP and pBAD-N48/GFP were constructed from p33N/GFP encoding KdpD-N and KdpD-N(Gly) in pBAD33. A 2.1 kb PstI-HindIII fragment from pBAD33 was used to amplify a 1.25 kb fragment from the plasmid pSF18 encoding KdpD-N (Facey and Kuhn, 2003). This fragment was digested with NdeI and PstI and cloned into the corresponding sites of pBAD18/GFP. To generate the plasmids, pSF163 and pSF165, the coding region of KdpD-N(Gly) was excised by XbaI-HindIII from pBAD-N25/GFP and pBAD-N48/GFP and inserted into the corresponding sites of pMS119EH, respectively. N22–48/GFP, containing amino acid residues 22–48 of KdpD fused to GFP, was constructed as follows: amino acids 2–21 were deleted by site-directed mutagenesis in pBAD-N48/GFP. The coding regions of all constructs were verified by sequence analysis.
**Fluorescence microscopy**

Strains were grown overnight at 37°C, diluted 1:20 in fresh LB medium, and grown to an OD_{600} of 0.4. IPTG was then added to a final concentration of 1 mM, and 0.2% (w/v) arabinose was added in strains carrying kdpD genes under the control of the araBAD promoter and operator. The cells were incubated for 30 min to 1 h at 30°C under continuous shaking. Rifampicin (1 mg ml\(^{-1}\), final concentration) was added, and the cultures were incubated at 30°C for the times indicated under Results. It was verified that under these conditions, the expression of the KdpD-GFP fusion proteins was inhibited. Cells were either collected by centrifugation, washed twice with LB medium and re-suspended in 2 mM EDTA, 50 mM Tris-HCl, pH 8.0 or treated with trichloroacetic acid (TCA) (10%, final concentration) and analysed by Western blotting. A cell suspension (3 μl) was applied to a polylysine-coated cover glass (Sigma-Aldrich) and the cells were examined immediately by Zeiss LSM 510 Meta, confocal fluorescence microscope. Emission was detected with a filter specific for GFP.

**Pulse-chase and immunoprecipitation analyses**

For all experiments, cells were grown to mid-logarithmic phase. Cells harbouring the plasmid-encoded proteins were induced for 10 min with IPTG (1 mM, final concentration). Cells were then labelled with 10 μCi ml\(^{-1}\) of [\(^{35}\)S]methionine for 1 min and chased with excess l-methionine for 2 min. For spheroplasting, cells were centrifuged and re-suspended in 15 min. Aliquots of the spheroplast suspension were incubated at 30°C for the times indicated under Results and EDTA (1 mM, final concentration) were added for 15 min. An OD_{600} of 0.5 was reached, the cells were induced either with IPTG (1.0 mM, final concentration) or arabinose (0.2%, final concentration). A lysis control was included by adding 2.5% Triton X-100 and proteinase K (0.5 mg ml\(^{-1}\), final concentration). Samples were precipitated with TCA (20%, final concentration), re-suspended in 10 mM Tris/2% SDS, pH 8.0 and immunoprecipitated with antibodies against HA, KdpD, OmpA (a periplasmic control), or GroEL (a cytoplasmic control). Samples were analysed by SDS-PAGE and phosphorimaging.

**Expression and purification of GFP fusion proteins**

*Escherichia coli* strain MC1061 harbouring the plasmid pGFPmut3.1 (Clontech) or pBAD-N25–GFP or pBAD-N48–GFP was grown at 30°C in LB medium (1 l) with 100 μg ml\(^{-1}\) ampicillin or 25 μg ml\(^{-1}\) chloramphenicol, respectively. When an OD_{600} of 0.5 was reached, the cells were induced either with IPTG (1.0 mM, final concentration) or arabinose (0.2%, final concentration), respectively, and grown for an additional 10 h at 30°C. Cells were harvested by centrifugation and pellets were re-suspended in 0.05 M sodium phosphate buffer, pH 7.5, 0.25 M NaCl and lysed with a French press. The cell debris was removed by centrifugation. The filtered extract was applied to IMAC column containing Cheleting Sepharose 6B resin (Pharmacia) charged with Ni(II) ions, which had been pre-equilibrated with 0.05 M sodium phosphate buffer, pH 7.5, 2 M NaCl as described by Li et al. (2001). Unbound protein was removed from the column by washing with 10 column volumes of equilibration buffer. Protein was eluted from the column with a falling pH (7.5–4.0) in a 0.05 M sodium phosphate buffer and 2 M NaCl. The purification process was monitored by SDS-PAGE analysis.

**Pull-down assays**

The purified His-SRP protein (Ffh + 4.5S RNA) was kindly provided by I. Sinning. For pull-down assays, about 5 μg of His-SRP protein was attached to a column containing 300 μl of Ni Sepharose High Performance (Amersham Biosciences) which had been pre-equilibrated with 1.5 ml of equilibration buffer (15 mM NaHPO\(_4\), 150 mM NaCl, 10% glycerol, 25 mM imidazole, pH 7.6) for 1 h at 4°C. Unbound protein was collected by centrifugation. GFP-fused proteins (~5 μg) were added to the immobilized His-SRP protein and were incubated for 1 h at 4°C. After extensive washing with 1.8 ml of equilibration buffer containing 25 mM imidazole, bound proteins were eluted with 500 mM imidazole. The expected size of the co-eluted proteins were verified by SDS-PAGE (15%) and Western blot analysis.

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