F_1F_0 ATP synthase subunit c is targeted by the SRP to YidC in the *E. coli* inner membrane

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Abstract ***Escherichia coli*** **inner membrane proteins (IMPs) use different pathways for targeting and membrane integration. We have examined the biogenesis of the F_1F_0 ATP synthase subunit c, a small double spanning IMP, using complementary in vivo and in vitro approaches. The data suggest that F_0c is targeted by the SRP to the membrane, where it inserts at YidC in a Sec-independent mechanism. F_0c appears to be the first natural substrate of this novel pathway.**

Keywords: ATP synthase; Signal recognition particle; YidC; Protein targeting; Membrane protein; *E. coli*

1. Introduction

Escherichia coli IMPs use distinct targeting, insertion and assembly factors to reach their native membrane embedded conformation. Most IMPs depend on a conserved system consisting of the signal recognition particle (SRP) and its receptor FtsY for efficient targeting to the membrane (reviewed in [1,2]). The SRP binds to hydrophobic targeting signals present in nascent IMPs. The ribosome nascent chain–SRP complex is then transferred to a membrane insertion site via FtsY [2].

Recently, YidC has been identified as an insertion or assembly factor that plays a critical role in the biogenesis of most IMPs (reviewed in [3,4]). YidC is a member of the Alb3/Oxa1/YidC protein family that appears involved in the biogenesis of membrane proteins in chloroplasts, mitochondria and bacteria [5]. Interestingly, *E. coli* YidC functions in two insertion pathways: (i) in physical and functional association with the Sec-translocon and (ii) in a separate homo- or hetero-oligomeric form. All tested Sec-dependent IMPs contact YidC during membrane insertion [6–9]. The contact with YidC is transient and specific for trans-membrane segments (TMs) of substrate proteins suggesting that YidC functions in the release of TMs from the Sec-translocon into the lipid bilayer [6]. In contrast, certain small phage coat proteins require only YidC for membrane integration, implying a more elaborate function for the "free" form of YidC in this integration pathway [10,11].

Remarkably, depletion of YidC has little effect on the efficiency and kinetics of IMP insertion via the Sec–YidC pathway as determined in protease accessibility experiments [12]. On the other hand, depletion of YidC almost completely blocks integration of phage coat proteins via the "YidC-only" pathway [11]. This raises the question why YidC is essential. With respect to this issue, we have recently shown that depletion of YidC strongly affects steady state levels of the important endogenous proteins CyoA (subunit II of the cytochrome *o* oxidase) and F_0c (subunit c of F_1F_0 ATPase) in the inner membrane [13].

Here, we have analyzed the mechanism of membrane targeting and integration of F_0c using a combined in vitro (cross-linking) and in vivo (protease mapping) approach. Combined, the results demonstrate that F_0c is co-translationally targeted by the SRP and inserted at YidC in the membrane with only a marginal role, if any, for the Sec-translocon. This unveils a novel pathway of IMP biogenesis of which F_0c is the first natural exponent.

2. Materials and methods

2.1. Strains, plasmids and growth conditions

The Ffh (fifty-four homolog) depletion strain HDB51 and the SecE depletion strain CM124 were grown as described [14,15]. Strain HTP406, which expresses a mutant *ffh* allele, and its isogenic control JP313 were grown as described [16]. The YidC temperature sensitive strain KO1671 and its isogenic control KO1670 were grown as previously described [17].

For in vivo protease accessibility assays, the *uncE* gene (encoding F_0c) was PCR amplified from *E. coli* genomic DNA. A HA-tag (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala), preceded by a flexible linker peptide (Pro-Gly-Gly), was attached to the C-terminus of F_0c by PCR. The PCR fragment was cloned into pBAD24 [18], pEH1 and pEH3 [19], and pASKIBA3 (IBA GmbH). For control experiments, M13P2 and FtsQ coding sequences were cloned in pASKIBA3.

For in vitro cross-link experiments, plasmid pC4Meth79F_0cTAG15 was constructed by PCR using genomic *E. coli* DNA as template. It encodes truncated F_0c fused to a C-terminal 4× methionine tag to improve labeling efficiency, and contains an amber mutation at position 15 to allow sup-tRNA photocross-linking, as described previously [6]. The nucleotide sequence of all constructs was confirmed by DNA sequencing.

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Abbreviations: Ffh, fifty-four homolog; IMP, inner membrane protein; IMV, inverted membrane vesicle; SRP, signal recognition particle; TF, trigger factor

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2.2. In vivo protease K accessibility assay

Cells harboring derivatives of pEH1, pEH3, pBAD24 or pASKIBA3 were induced for 10 min by adding IPTG (pEH1 and pEH3; 1 mM), L-arabinose (pBAD24; 0.2%) or anhydrotetracycline (pASKIBA3; 500 ng/ml), labeled with [^{35}S]methionine (30 μCi/ml), converted to spheroplasts and processed as described previously [12,17].

2.3. In vitro cross-linking

Preparation of truncated mRNA, in vitro translation, targeting to inverted membrane vesicles (IMVs), photocross-linking, carbonate extraction and sample processing were performed as described previously [6].

3. Results

3.1. SRP-mediated targeting of F_0c to the inner membrane

F_0c is a small, double spanning membrane protein with a short N- and C-terminus exposed to the periplasm ([20]; Fig. 1). We have recently shown that the presence of this protein in the membrane is atypically sensitive to depletion of YidC [13], which prompted us to study the mechanism of targeting and insertion of F_0c in detail. To permit immunodetection in protease accessibilty experiments, a HA-tag was added to the periplasmic C-terminus of F_0c (Fig. 1). F_0c-HA fully restored growth of the *uncE114* mutant strain MM944I^q [21] on minimal medium plates containing succinate (data not shown), indicating that the HA tag does not interfere with assembly and activity of the F_1F_0 ATPsynthase.

We initially analyzed the role of the SRP in targeting of F_0c. The SRP is composed of the signal binding protein Ffh and the 4.5S RNA [2]. F_0c-HA was expressed in strain HDB51 in which *ffh* expression is under control of an arabinose-inducible promoter. Cells grown in the presence or absence of arabinose were pulse-labeled, converted to spheroplasts and treated with proteinase K to degrade external (periplasmic) protein (-domains). When Ffh was present, F_0c-HA was only detected in mock-treated samples (Fig. 2A). This confirms that the HA-tag in F_0c-HA is translocated and sensitive to proteinase K, validating our assay conditions. In contrast, upon depletion of Ffh, F_0c-HA was to a large degree protected against protease treatment suggesting that Ffh is required for efficient targeting or assembly of F_0c-HA to the membrane.

In this experiment, Ffh levels were reduced approximately threefold as judged by immunoblot analysis, whereas YidC levels were not affected (data not shown). Notably, expression and processing of the Sec-dependent OmpA protein were not affected under these conditions (Fig. 2A). Together, these results argue that the extent of depletion of the essential Ffh protein has not indirectly affected the functioning of the Sec/YidC machinery.

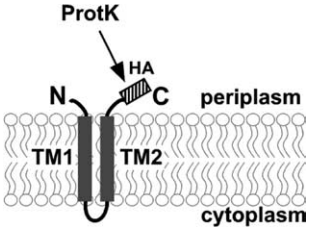

Fig. 1. Topological model of F_0c-HA. An HA-epitope was attached to the C-terminus of F_0c to facilitate verification of membrane integration. In spheroplasts derived from wild-type cells, proteinase K degrades the translocated HA epitope of F_0c-HA.

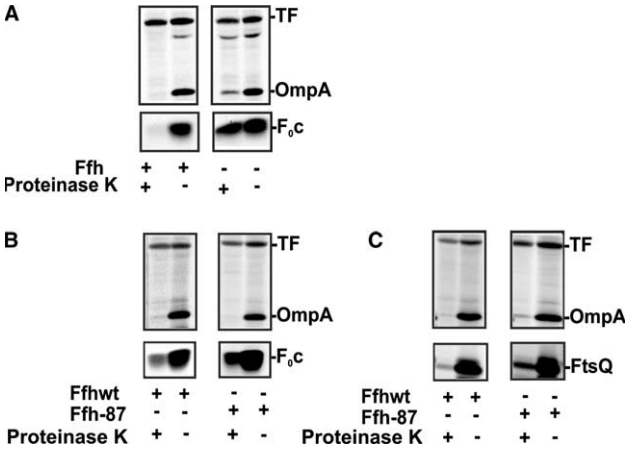

Fig. 2. F_0c requires the SRP for membrane targeting. A protease accessibility assay was used to monitor the effects of depletion (A) or mutation (B) of Ffh on biogenesis of F_0c-HA. Strain HDB51 harboring a plasmid expressing F_0c-HA was grown under Ffh depleting or non-depleting conditions (A). The Ffh mutant strain HTP406 was grown in parallel with its isogenic parent strain JP313 (B, C). HTP406 and JP313 carried the F_0c-HA plasmid or, as a control, the FtsQ expression plasmid (C). Cells were pulse-labeled and processed as described in Section 2. Anti-HA serum was used for immunoprecipitation of F_0c-HA and antibodies against the N-terminus of FtsQ were used for immunoprecipitation of FtsQ. Immunoprecipitated material was subjected to SDS–PAGE and phosphorimaging. TF is a cytoplasmic control protein that is not accessible to protease in intact spheroplasts. OmpA is an outer membrane protein that is susceptible to protease in spheroplasts and serves to monitor the effciency of spheroplast formation.

A possible role of the SRP in F_0c targeting was further investigated in the relatively mild and viable mutant Ffh-87 strain that has recently been described [16]. Consistent with the effects of Ffh depletion, this mutation caused significant accumulation of F_0c-HA in proteinase K treated spheroplasts, whereas OmpA processing was not affected (Fig. 2B). As an additional control, insertion of the SRP-dependent IMP FtsQ was analyzed in the Ffh-87 strain. The reduction in accessibility of this protein in the Ffh-87 strain was similar to the effect on F_0c-HA confirming both proteins require the SRP for efficient targeting ([16]; Fig. 2C).

The SRP interacts with the first TM of most nascent IMPs. We investigated the molecular environment of TM1 in nascent F_0c by site-specific photo cross-linking. Using suppressor-tRNA technology, a cross-linking probe was introduced at position 15 in the hydrophobic core of TM1 during in vitro synthesis of F_0c. The construct used for F_0c expression was 79 amino acids long, equal to the full-length protein but lacking a stop codon to terminate translation. Consequently, nascent (ribosome-associated) F_0c was produced with TM1-containing the photoreactive probe-exposed outside the ribosome (Fig. 3A). Activation of the probe by UV-irradiation generated a prominent cross-linking product at ~55 kDa that could be immmunoprecipitated using anti-Ffh serum (Fig. 3A, lane 3) suggesting association of TM1 with the SRP. Notably, Ffh was cross-linked in a wild-type translation lysate in which the concentration of Ffh is very low suggesting that TM1 in nascent F_0c has a high affinity for Ffh. Other products of lower intensity were observed in which trigger factor (TF) and the ribosomal protein L23 could be identified as cross-linking partners (Fig. 3A, lanes 2 and 4). L23 is located near the na-

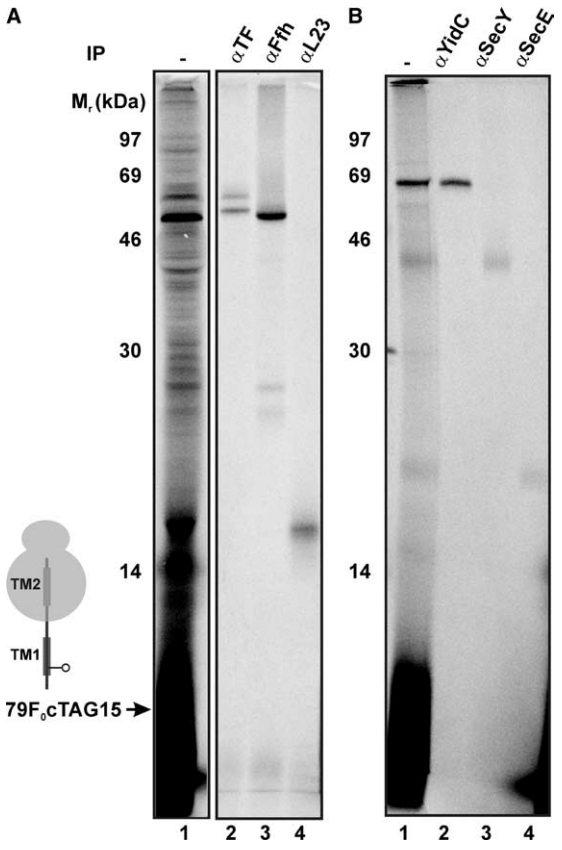

Fig. 3. Interactions of nascent F_0c. In vitro synthesis of nascent $79F_0c$ with a TAG codon at position 15 was carried out in the presence of (Tmd)Phe-$tRNA^{Sup}$ either in the absence (A) or presence (B) of *E. coli* IMVs. After translation, samples were UV-irradiated and TCA precipitated (A), or extracted with sodium carbonate (B). TCA precipitates and carbonate pellet fractions were immunoprecipitated using antisera against the indicated proteins (lanes 2–4), or directly analyzed by SDS–PAGE (lane 1).

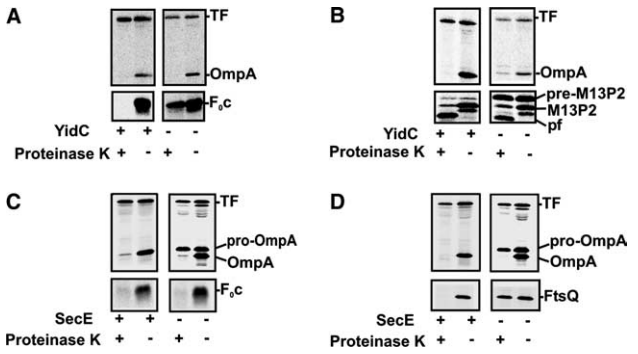

Fig. 4. F_0c-HA requires YidC but not the Sec-translocon for membrane insertion. A protease accessibility assay was used to monitor the effects of depletion of YidC (A,B) or SecE (C, D) on biogenesis of F_0c-HA. The YidC temperature sensitive mutant KO1671 and its control parent strain KO1670 were grown at the non-permissive temperature (42 °C). KO1671 and KO1670 harbored the F_0c-HA plasmid (A) or, as a control, the M13P2 expression plasmid (B). Cells were pulse-labeled and processed as described in Section 2. Anti-HA serum was used for immunoprecipitation of F_0c-HA and antibodies against Lep were used for immunoprecipitation of M13P2. Immunoprecipitated material was subjected to SDS–PAGE and phosphorimaging. M13P2 is synthesized with a signal peptide (pre-M13P2) that is processed during membrane integration to yield mature M13P2. In proteinase K-treated spheroplasts, mature M13P2 is converted to a slightly smaller form, denoted pf (proteinase K fragment). The SecE depletion strain CM124 was grown under SecE depleting or non-depleting conditions. CM124 carried the F_0c-HA plasmid (C) or, as a control, the FtsQ expression plasmid (D) Antibodies against the N-terminus of FtsQ were used for immunoprecipitation of FtsQ.

scent chain exit site and trigger factor is a cytosolic chaperone with a general affinity for nascent polypeptides [3]. Both Ffh and TF have been shown to associate with L23 [22–24].

Considered together, the data strongly suggest that the SRP plays a crucial role in targeting of F_0c to the inner membrane.

3.2. YidC-mediated insertion of F_0c into the inner membrane

Next, the requirements for membrane insertion of F_0c-HA were analyzed. Depletion of YidC strongly inhibited proteolysis of F_0c-HA as compared with the non-depleted (control) cells (Fig. 4A). A similar strong effect was observed on the processing and protease accessibility of the M13P2 hybrid protein that served as a YidC-dependent/Sec-independent control IMP ([12]; Fig. 4B). Translocation of OmpA appeared unaffected demonstrating that indirect inactivation of the Sec-translocon had not occurred under these conditions (Fig. 4A and B; [13]).

To determine the role of the Sec-translocon in F_0c insertion we used strain CM124 in which the essential *secE* gene is under control of an arabinose-inducible promoter. Depletion of SecE results in the loss of the complete SecY/E core of the translocon since SecY is rapidly degraded in the absence of SecE [25]. Clearly, depletion of SecE had no discernible effect on the accessibility of F_0c-HA towards proteinase K (Fig. 4C). In contrast, proteolysis of FtsQ was almost completely blocked under these conditions (Fig. 4D) confirming earlier results [26]. Also, processing and translocation of OmpA were strongly reduced as expected for this Sec-dependently translocated protein (Fig. 4C and D).

To obtain independent information on the mode of insertion of F_0c, we repeated the cross-linking assay described above, but added IMVs during translation to allow membrane targeting and insertion. Subsequently, cross-linking was induced by UV-irradiation and membrane integrated material was recovered by extraction with sodium carbonate. An unambiguous and almost exclusive cross-linking product was detected at ~69 kDa that represents cross-linking to YidC as shown by immunoprecipitation (Fig. 3B, lane 2). Weak products at ~44 kDa and ~20 kDa represent cross-linking to SecY and SecE, respectively (Fig. 3B, lanes 3 and 4). This confirms that the TM1 in nascent F_0c is primarily in contact with YidC during membrane insertion.

Together, the data suggest that F_0c utilizes a YidC-dependent but Sec-independent mechanism for membrane insertion.

4. Discussion

F_0c is a small membrane protein with two TMs in an N_{out}–C_{out} topology [20]. 10 F_0c subunits assemble in an oligomeric ring structure that together with 1 F_0a and 2 F_0b subunits form the membrane embedded F_0 complex [27]. Recent studies suggest that YidC plays a role in the biogenesis of this complex [13,28]. Using complementary in vitro and in vivo approaches we show here that YidC can function as the insertase for F_0c, independent of the Sec-translocon. Insertion of

F_0c is strongly affected by YidC depletion in a pulse-label experiment, whereas depletion of the essential translocon component SecE had no discernible effect (Fig. 4A and C). Furthermore, in vitro photocross-linking of nascent F_0c shows prominent cross-linking of its first TM to YidC and only weak cross-linking to SecY and SecE (Fig. 3B). The latter contacts may represent a subpopulation of nascent chains that uses an alternative integration pathway that involves the Sec-translocon, if present. In essence, our data are compatible with those of van der Laan and coworkers who, in the course of this study, reported on the insertion of F_0c in proteoliposomes that contain only YidC [29]. Combined, the data are reminiscent of the YidC-mediated, Sec-independent mechanism of insertion of the small phage coat proteins M13 [30] and Pf3 [31].

How is F_0c targeted to YidC? Our data suggest a prominent role for the SRP in this process. TM1 in nascent F_0c is strongly cross-linked to Ffh from a wild-type lysate (Fig. 3A, lane 3) in which Ffh is present in very low concentrations. This assay has proven diagnostic in the determination of targeting pathway selection [32–34]. Indeed, depletion of Ffh or 4.5S RNA, the two constituents of the SRP, had a pronounced effect on the insertion of F_0c in vivo (Fig. 2A and data not shown). Although the export of the secreted SecB-dependent outer membrane protein OmpA was not affected under these conditions, indirect effects due to an altered cell physiology cannot be entirely excluded. However, similar results were obtained in a strain that displays only weak effects on the SRP pathway by a point mutation in *ffh* and that has relatively normal growth rates (Fig. 2B). In marked contrast, Van der Laan et al. [29], observed only small effects of depletion of Ffh or FtsY on F_0c insertion in a semi-reconstituted in vitro system. At present, we do not know the reason for this discrepancy. Noticeably, co-translational addition of membrane vesicles is a prerequisite for in vitro insertion of F_0c ([29], our unpublished data) which is difficult to reconcile with a SRP-independent, post-translational targeting mechanism as described for the phage coat proteins M13 and Pf3 [30,31].

Based on the data presented, F_0c appears to be the first natural substrate of a novel biogenesis route in which the SRP delivers the nascent IMP at YidC that catalyzes insertion in a seemingly Sec-independent mechanism. Co-translational targeting to YidC might facilitate and coordinate lipid partitioning of the two TMs in nascent F_0c preventing erroneous intra- and intermolecular contacts due to their hydrophobic nature. The existence of an SRP/YidC pathway has been suggested before based on the exclusive SRP and YidC dependence of an artifical IMP construct [12]. Interestingly, in thylakoids, a cpSRP/Alb3 pathway is operational and used by LHCP [35]. A functional interaction between cpSRP, cpFtsY and Alb3 was demonstrated [36], suggesting that similar interactions may contribute to targeting to YidC in *E. coli*. Future studies will concentrate on the mechanism and substrate specificity of this SRP/YidC pathway. During preparation of this manuscript, we learned of the work of Yi et al. [37]. There are interesting points of comparison with the latter paper which may need to be addressed in the future.

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