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Detection of cross-links between FtsH, YidC, HflK/C suggests a linked role for these proteins in quality control upon insertion of bacterial inner membrane proteins

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Abstract **Little is known about the quality control of proteins upon integration in the inner membrane of *Escherichia coli*. Here, we demonstrate that YidC and FtsH are adjacent to a nascent, truncated membrane protein using in vitro photo cross-linking. YidC plays a critical but poorly understood role in the biogenesis of *E. coli* inner membrane proteins (IMPs). FtsH functions as a membrane chaperone and protease. Furthermore, we show that FtsH and its modulator proteins HflK and HflC copurify with tagged YidC and, vice versa, that YidC copurifies with tagged FtsH. These results suggest that FtsH and YidC have a linked role in the quality control of IMPs.**

Structured summary:

MINT-6478034:

hflB (uniprotkb:P0AAI3) *physically interacts* (MI:0218) with *yidC* (uniprotkb:P25714), *hflK* (uniprotkb:P0ABC7), *hflC* (uniprotkb:P0ABC3) by *cross-linking studies* (MI:0030)

MINT-6478363:

yidC (uniprotkb:P25714) *physically interacts* (MI:0218) with *lldD* (uniprotkb:P33232), *rplD* (uniprotkb:P60723), *yrbD* (uniprotkb:P64604), *rpsA* (uniprotkb:P0AG67), *aphA1* (uniprotkb:P00551), *dacC* (uniprotkb:P08506), *rpsC* (uniprotkb:P0A7V3), *rpsD* (uniprotkb:P0A7V8), *rpsE* (uniprotkb:P0A7W1), *rpoA* (uniprotkb:P0A7Z4), *ompC* (uniprotkb:P06996), *ompA* (uniprotkb:P0A910), *atpA* (uniprotkb:P0ABB0), *atpD* (uniprotkb:P0ABB4), *adhE* (uniprotkb:P0A9Q7), *hflB* (uniprotkb:P0AAI3), *hflC* (uniprotkb:P0ABC3), *hflK* (uniprotkb:P0ABC7), *lacI* (uniprotkb:P03023), gapA (uniprotkb:P0A9B2), *rbsB* (uniprotkb:P02925), *sdhA* (uniprotkb:P0AC41), *rho* (uniprotkb:P0AG30), *udp* (uniprotkb:P12758), *nuoC* (uniprotkb:P33599), *treB* (uniprotkb:P36672) and *manX* (uniprotkb:P69797) by *cross-linking studies* (MI:0030)

MINT-6477988:

yidC (uniprotkb:P25714) *physically interacts* (MI:0218) with *hflB* (uniprotkb:P0AAI3), *hflK* (uniprotkb:P0ABC7), *hflC* (uniprotkb:P0ABC3), *secD* (uniprotkb:P0AG90) and *secG* (uniprotkb:P0AG99) by *cross-linking studies* (MI:0030)

MINT-6478012:

yidC (uniprotkb:P25714) *physically interacts* (MI:0218) with *hflC* (uniprotkb:P0ABC3) and *hflK* (uniprotkb:P0ABC7) by *cross-linking studies* (MI:0030)

Keywords: FtsH; HflK; HflC; SecYEG; YidC

1. Introduction

Recent biochemical and structural evidence has provided detailed insight in the targeting and insertion of bacterial membrane proteins. In comparison, later stages in the biogenesis of membrane proteins like the exit from the translocon, folding, oligomerization and quality control are less well characterized. *Escherichia coli* YidC and FtsH have been implicated in these late stages although their precise contribution is unclear.

YidC belongs to a novel class of facilitators of membrane protein assembly that includes homologues in mitochondria and chloroplasts [1]. Remarkably, YidC acts both in concert with and distinct from the SecYEG-translocon, the protein conducting channel that is used for translocation of secretory proteins and for insertion of inner membrane proteins (IMPs) [2,3]. Independent from the Sec-translocon, YidC catalyzes insertion of relatively simple IMPs. Sec-dependent IMPs were cross-linked to YidC at various stages during synthesis and membrane insertion suggesting a role of YidC in the recognition, lateral transfer and assembly of transmembrane segments (TMs) on their way from the Sec-translocon into the lipid bilayer [1]. Finally, YidC has been implicated in the folding of IMPs based on impaired in vitro folding of LacY in YidC depleted membrane vesicles and the upregulation of stress response pathways that react to impaired membrane protein folding upon depletion of YidC in vivo [4,5].

FtsH is known to degrade a subset of misassembled IMPs thus contributing to quality control [6]. It is an ATP-dependent, processive endopeptidase that is able to dislocate substrates from the membrane prior to their degradation by the

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Abbreviations: IMP, inner membrane protein; TM, transmembrane segment

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cytosolic protease domain. In addition, FtsH degrades certain cytosolic proteins and is thought to possess chaperone-like properties. FtsH is found in large complexes with HflK and HflkC, both IMPs with a large periplasmic domain, that are thought to repress the proteolytic activity of FtsH. Interestingly, circumstantial evidence suggests that FtsH also functions in membrane insertion and translocation. Depletion of FtsH retards translocation of secretory proteins and enhances the translocation of a cytosolic reporter domain of an artificial membrane protein.

Here, we present evidence that YidC is connected with FtsH suggesting a linked role for these proteins in quality control.

2. Materials and methods

2.1. Reagents, enzymes and sera

Detailed information about the reagents, enzymes and sera is given in the Supplementary material.

2.2. Strains, plasmids and growth conditions

YidC depletion strain JS7131 was used for complementation experiments [2]. *E. coli* strain Top10F' (Invitrogen) was used for expression of YidC(-derivatives) and for routine cloning and maintenance of plasmid constructs. Plasmids pC4Meth55BRPTAG10 and pCL1921-YidC.K_m have been described previously [7,8]. The strains were routinely grown in Luria Bertani (LB) medium with appropriate antibiotics. For YidC co-purification experiments, the *yidC* gene was PCR amplified from pEH1-YidC [9] using a reverse primer that included the codons for the StrepTagII (WSPQFEK) preceded by a linker peptide (SA). The resulting YidC-Strep PCR fragment was cloned into pCL1921.K_m [10] yielding pCL1921-YidC-Strep.K_m. For FtsH co-purification experiments, the *ftsH* gene was PCR amplified from *E. coli* K12 genomic DNA, using a reverse primer that included the codons for the StrepTagII (WSPQFEK) preceded by a linker peptide (SA). The resulting FtsH-Strep PCR fragment was cloned into pCL1921.K_m [10] yielding pCL1921-FtsH-Strep.K_m. The nucleotide sequences of all constructs were verified by DNA sequencing.

2.3. In vitro cross-linking

Preparation of truncated mRNA, in vitro translation of nascent BRP and Lpp, targeting to inverted inner membrane vesicles (IMVs), photo cross-linking, carbonate extraction and sample processing were performed as described previously [3].

2.4. Affinity purification of YidC complexes and identification by mass spectrometry

YidC containing complexes were affinity purified and characterized by mass spectrometry as described in the Supplementary material.

2.5. Affinity purification of FtsH complexes

FtsH containing complexes were affinity purified as described in the Supplementary material.

3. Results

3.1. Nascent BRP simultaneously crosslinks to YidC and FtsH

Using in vitro photo cross-linking we have previously demonstrated that YidC contacts TMs in various Sec-dependent IMPs during biogenesis. In all tested cases, cross-linking to YidC was reduced upon release of the nascent IMP from the ribosome by puromycin or EDTA suggesting that the contact with YidC is dependent on the context of the ribosome. In principle, detached nascent chains in the membrane may be subject to quality control mechanisms as they represent truncated, probably misassembled membrane proteins. Therefore, cross-linking partners of these released chains potentially recognize these features and might play a role in quality control. For instance, upon release from the ribosome, nascent membrane-integrated leader peptidase (Lep) is transferred from YidC to a ~56 kDa protein that remains to be identified [11]. Secondly, we reported that cross-linking of an unidentified ~70 kDa protein to the signal peptide of nascent bacteriocin release protein (BRP), a membrane lipoprotein, increased upon EDTA treatment [7]. Notably, the latter cross-linked product was observed in carbonate-extracted samples, suggesting that it is membrane-integrated, together with cross-linked YidC (and to a lesser extent SecY and SecA). Here, we have essentially repeated this experiment and identified the 70 kDa cross-linked partner as FtsH by immunoprecipitation (Fig. 1). Like EDTA [7] addition of puromycin prior to cross-linking caused a shift in cross-linking from YidC to FtsH. Similar but weaker cross-linking to both YidC and FtsH was observed for nascent murein lipoprotein (data not shown). Importantly, in these cross-link experiments wild-type membrane vesicles were used that contain endogenous levels of YidC and FtsH. Together, the data suggest that YidC and FtsH act sequentially in the quality control of a subset of membrane proteins.

3.2. Affinity purification of YidC

The simultaneous cross-linking and apparent transfer of substrate from YidC to FtsH implies that these factors are in close proximity or even associate during membrane insertion of certain proteins. To examine a physical connection between YidC and FtsH, we affinity purified YidC under relatively mild

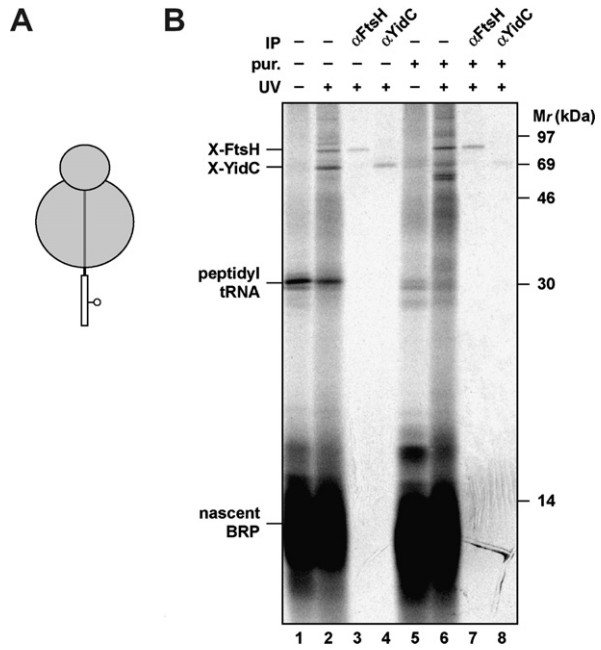

Fig. 1. Nascent BRP simultaneously crosslinks to YidC and FtsH. (A) Schematic representation of the 55BRP construct with a cross-linking probe at position 10 (55BRPTAG10). The signal sequence is presented as a white bar. (B) 55BRPTAG10 was translated in the presence of IMVs and (Tmd)-Phe-tRNAsup as described in Section 2. After translation, the nascent chains were treated with 2 mM puromycin when indicated, UV irradiated or kept in the dark, and subsequently extracted with sodium carbonate (lanes 1–2; 5–6). UV-irradiated membrane fractions were immunoprecipitated using antiserum against FtsH and YidC (lanes 3–4; 7–8).

conditions allowing an unbiased assessment of copurifying factors. An expression vector was constructed encoding YidC containing a C-terminal StrepTagII (YidC-Strep). The presence of the tag did not interfere with the activity of YidC as judged by the ability of YidC-Strep to complement growth of the YidC depletion strain JS7131 (data not shown) and is consistent with the previous observation that the C-terminus of YidC is not essential for function [12]. To identify proteins that co-purify with YidC-Strep, total membranes were isolated from cells in which YidC-Strep is moderately overexpressed (5- to 10-fold) and treated with the membrane-permeable, homobifunctional (lysine-specific) thiol-cleavable, chemical cross-linker DSP to fix protein interactions. Subsequently, the membranes were solubilized using the relatively mild detergent DDM to preserve the integrity of membrane protein complexes and subjected to a one-step affinity purification using StrepTactin-Sepharose. The eluates from this step were incubated with DTT to break the cross-links and analyzed by SDS–PAGE and immuno blotting (Fig. 2A). YidC was specifically detected in the eluates of cross-linked or mock-treated YidC-Strep membranes. As a control, untagged YidC expressed in parallel, was not eluted under these conditions (Fig. 2A) but was recovered in the flow-through fraction (not shown) confirming that YidC does not a specifically bind to the affinity resin. Strikingly, FtsH was exclusively detected in the eluate of cross-linked YidC-Strep membranes. More-

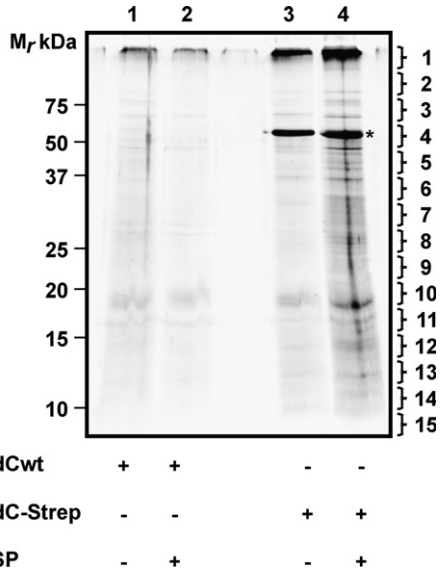

Fig. 3. Characterization of co-purifying proteins. Following DSP-crosslinking of membranes and affinity purification by a mild procedure as described in Section 2 the eluates of wild-type YidC (YidCwt) (lanes 1 and 2) and strep-tagged YidC (lanes 3 and 4) were subjected to SDS–PAGE. Proteins were visualized by MS compatible silver staining and lanes of interest (lanes 2 and 4) were excised and cut in 15 pieces followed by trypsin digestion and peptides were identified by MS (Table 1). The position of strep-tagged YidC is indicated by an asterisk.

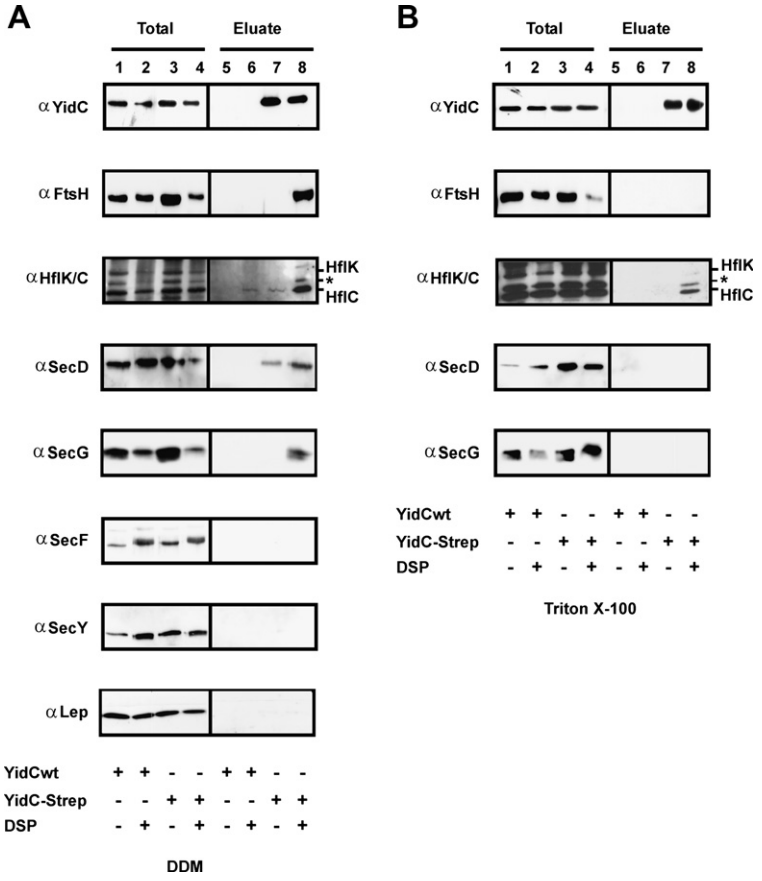

Fig. 2. Immunoblot analysis of co-purifying proteins. Total membranes derived from cells expressing wild-type YidC (YidCwt) or strep-tagged YidC (YidC-Strep) were treated with the cross-linker DSP and processed as described in Section 2. One part was subjected to affinity purification by a mild procedure (A). The other part was subjected to affinity purification by a stringent procedure (B). Ten percent of total (lanes 1–4) and 100% of eluate (lanes 5–8) were analyzed by immunoblotting with the indicated antibodies. A non-specific protein cross reacting with antiserum against HflK and HflC is indicated by an asterisk. Black lines indicate that intervening lanes have been spliced out.

over, HflK and HflC were co-eluted in the same fraction. As a control for the specificity of the procedure, the unrelated IMP, Lep (Fig. 2A) was not detected in any of the eluate samples. Together, the data suggest that at least a fraction of YidC-Strep is in close proximity to or even interacts with the FtsH/HflK/C complex in the inner membrane under steady state conditions suggesting a functional affiliation. It is formally possible that the FtsH/HflK/C complex is recruited by misassembled YidC-Strep. However, pulse-chase analysis revealed that YidC-Strep is very stable at the expression level used making this explanation less likely (data not shown).

Interestingly, it has been shown by co-immunoprecipitation that FtsH is not only associated with HflK/C but also with unidentified membrane proteins of 31, 60 and 62 kDa [13]. Though speculative, YidC (60 kDa) might have been co-purified in this study.

Previously, YidC has been co-purified with overproduced Sec-translocase complexes [3,14,15]. Here, we have analyzed co-elution of Sec subunits (endogeneously expressed) with YidC-Strep. SecD and SecG but not SecF and SecY were detectably co-purified. SecE elution could not be assessed because of poor serum quality. Notably, SecD even co-purified to some extent with YidC-Strep from uncross-linked membranes suggesting a relatively stable interaction [15].

The combination of cross-linking and mild detergent-solubilization (DDM, low-salt) of membranes described above does not distinguish between proteins that are directly or indirectly associated and cross-linked to YidC-Strep. In an attempt to remove proteins not directly cross-linked to YidC we also analyzed eluates of YidC-Strep derived from membranes that were solubilized by a more stringent procedure (Triton X-100, high-salt) (Fig. 2B). Under these conditions HflK/C, but not FtsH, SecD and SecG were co-purified with YidC-Strep indicating that the association of FtsH may be indirect via HflK/C. Apparently, SecD and SecG are not directly cross-linked to YidC-Strep either.

3.3. Mass spectrometric characterization of co-purifying proteins

The identity of cross-linked and specifically co-purifying proteins was also determined in a more unbiased approach by peptide mass fingerprinting using in-gel digestion with tryp-

Table 1
Identification of co-purifying proteins

Gene	SwissProt accession #	Peptides	Localization[a]	Gene product	Gel slice
Transcriptional regulators					
LacI	P03023	3	Cyto	Lactose operon repressor	5
Rho	P0AG30	1	Cyto	Transcription termination factor rho	4
Translation					
RpsA	P0A667	1	Cyto	30S ribosomal protein S1	3
RpsC	P0A7V3	1	Cyto	30S ribosomal protein S3	8
RpsD	P0A7V8	3	Cyto	30S ribosomal protein S4	7,8,10
RpsE	P0A7W1	2	Cyto	30S ribosomal protein S5	12
RplD	P60723	1	Cyto	50S ribosomal protein L4	10
RpoA	P0A7Z4	3	Cyto	DNA-directed RNA polymerase alpha chain	5
Protease and regulators					
HflB	P0AAI3	1	IM, integral	Cell division protease FtsH	3
HflC	P0ABC3	1	IM, integral	Protein hflC	6
HflK	P0ABC7	0[c]	IM, integral	Protein hflK	5
Transport					
OmpA	P0A910	2	OM	Outer membrane protein A	6
OmpC	P06996	1	OM	Outer membrane protein C	5
Detoxification and protection					
aphA1	P00551	1	Cyto	Kanamycin kinase, type I	7
DacC	P08506	1	IM, peripheral	Penicillin-binding protein 6	5
Metabolism and energy production					
AtpA	P0ABB0	4	IM, peripheral	ATP synthase subunit alpha	4
AtpD	P0ABB4	4	IM, peripheral	ATP synthase subunit beta	4
AdhE	P0A9Q7	3	Cyto	Aldehyde–alcohol dehydrogenase	2
NuoC	P33599	2	IM, peripheral	NADH-quinone oxidoreductase chain C/D	3
SdhA	P0AC41	1	IM, peripheral	Succinate dehydrogenase flavo protein subunit	4
LidD	P33232	2	IM, peripheral	L-lactate dehydrogenase	5
TreB	P36672	1	IM, integral	IPTS system trehalose-specific EIIBC component	4
ManX	P69797	2	IM, peripheral	PTS system mannose-specific EIIAB component	6
Udp	P12758	1	Cyto	Uridine phosphorylase	8
RbsB	P02925	1	Peri	D-ribose-binding periplasmic protein	8
GapA	P0A9B2	2	Cyto	Glyceraldehyde-3-phosphate dehydrogenase A	6
Unknown function					
YrbD	P64604	1	Cyto[b]	Hypothetical protein yrbD	10

[a]Cellular localization is according to the SwissProt database (cyto, cytoplasm; peri, periplasm; IM, inner membrane; OM, outer membrane).
[b]The localization of unknown proteins was predicted using PSLpred.
[c]No peptides were detected by LC-ESI-MS/MS. However, an ion most likely derived of HflK was detected.

sin of complete lanes of DSP treated YidC-Strep and untagged YidC eluates (Fig. 3) followed by nano LC-ESI-MS/MS analysis. This procedure identified 27 *E. coli* proteins that were specifically recovered with YidC-Strep and grouped into functional categories based on annotations. They include a variety of regulatory proteins, ribosomal proteins and abundant cytosolic and peripheral membrane proteins with a reported role in metabolism and energy production (Table 1). Their relationship with YidC is not immediately obvious and they were not further considered in this study. Only four of the co-purifying proteins were recognized as integral IMPs. Strikingly these comprise FtsH, HflK and HflC. Furthermore, FtsH, HflK and HflC were also specifically co-purified with YidC-Strep in an independent initial experiment in which silver-stained bands rather than whole gel lanes were excised and analyzed by peptide mass fingerprinting (data not shown). Subunits of the Sec-translocon were not identified in the YidC-Strep eluates by this method. It should be noted that the MS procedure chosen is relatively ineffective for hydrophobic membrane proteins where protease sites are less frequent [16].

3.4. Affinity purification of FtsH

To further address a possible connection between FtsH and YidC, we performed a preliminary reciproke co-purification experiment using Strep-tagged FtsH (FtsH-Strep) as bait protein. Total membranes were isolated from cells in which FtsH-Strep was moderately overexpressed. Following DSP cross-linking, the membranes were solubilized by Triton X-100 and subjected to a stringent affinity purification procedure. Samples were eluted, treated with DTT to cleave cross-links and subjected to SDS–PAGE and immunoblotting with the indicated antisera (Fig. 4).

FtsH-Strep was specifically detected in the eluates of cross-linked or mock-treated samples. As expected, HflK and HflC were recovered in the same fractions. These proteins even co-purified to some extent from mock-treated samples, in agreement of a stable interaction between HflK, HflC and FtsH [13]. Importantly, YidC co-eluted with cross-linked FtsH-strep. To verify the specificity of the procedure, the IMPs SecG and SecY were not recovered in the eluate samples. In conclusion, these data confirm an interaction between YidC and the FtsH complex.

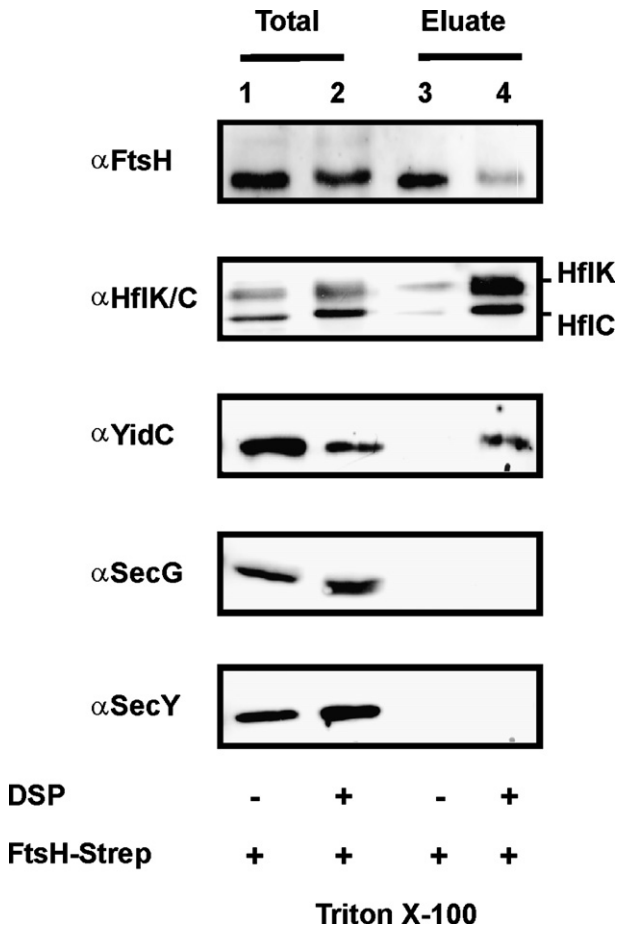

Fig. 4. FtsH interacts with YidC. Total membranes derived from cells expressing Strep-tagged FtsH (FtsH-Strep) were treated with the cross-linker DSP. Following solubilization of the membranes with Triton X-100, the samples were subjected to affinity purification by a stringent procedure. Samples were eluted, treated with DTT to cleave cross-links and subjected to SDS–PAGE and immunoblotting with the indicated antisera. Ten percent of total (lanes 1 and 2) and 100% of eluate (lanes 3 and 4) were loaded.

4. Discussion

Two independent approaches suggest that YidC is physically and functionally connected with the FtsH chaperone and protease complex. First, simultaneous cross-linking of both YidC and FtsH was observed to the signal peptide of a nascent membrane protein. Second, FtsH and its modulating factor HflK/C was co-purified with tagged YidC expressed at a moderate level and, vice versa, YidC was co-purified with tagged FtsH.

The copurification of FtsH and HflK/C with YidC suggests that YidC contributes to this quality control complex. In this context, YidC might select certain nascent membrane proteins upon their exit from the Sec-translocon and present them to FtsH for degradation. Presumably, stalled membrane proteins are harmful and require elimination. In contrast to YidC, FtsH remains associated with the nascent chains that have been released from the ribosome consistent with its late function in membrane protein biogenesis. Until now, only nascent BRP has been efficiently cross-linked to FtsH which may reflect the reported narrow substrate specificity of FtsH [6] or the peculiar properties of the BRP in combination with its stalled nature in the assay used. BRP is a small lipoprotein located in the inner and outer membrane that is required for secretion of the bacteriocin cloacinDF13 across the cell envelope [17]. Notably, the BRP contains a very hydrophobic signal peptide that mediates insertion via the SRP/Sec/YidC pathway and appears stable after cleavage from the mature protein [7]. Degradation of more labile IMP intermediates may preclude detection of their interaction with FtsH.

It is of interest to note that in mitochondria homologues of YidC and FtsH have been implicated in a similar partnership. Oxa1, the mitochondrial YidC homologue, has been selected in a genetic screen designed to identify multi-copy suppressors of the mitochondrial FtsH homologues Yta10 and Yta12 [18]. Furthermore, deletion of Yme1, another FtsH homologue, in an *oxa1* null mutant restores the level of the Oxa1 substrate F1Fo-ATP synthase to wt levels [19]. Based

on these observations, Oxa1 has been proposed to function as a chaperone that protects newly synthesized membrane proteins from degradation by FtsH-like proteases until they are properly folded. In *E. coli*, YidC might perform a similar function, most likely in the context of the Sec-translocon. Consistently, the IMP LacY is inserted but degraded upon depletion of YidC [4]. Furthermore, YidC deficiency was recently shown to elicit extracytoplasmic stress responses that have been attributed to the accumulation of malfolded IMPs [5]. Together, the data suggest a role for YidC in the quality control of IMPs during biogenesis. The precise functional and structural relationship between YidC and FtsH is a topic of ongoing research.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.02.082.

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