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The AAA+ FtsH protease degrades an ssrA-tagged model protein in the inner membrane of E. coli

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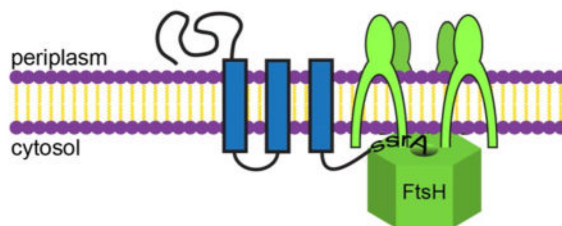
Abstract

In eubacteria, the tmRNA system frees ribosomes that stall during protein synthesis and adds an ssrA tag to the incompletely translated polypeptide to target it for degradation. The AAA+ ClpXP protease degrades most ssrA-tagged proteins in the *E. coli* cytoplasm and was recently shown to degrade an ssrA-tagged protein in the inner membrane. However, we find that tmRNA-mediated tagging of *E. coli* $ProW_{1\text{-}182}$, a different inner-membrane protein, results in degradation by the membrane-tethered AAA+ FtsH protease. ClpXP played no role in degradation of $ProW_{1\text{-}182}$ *in vivo*. These studies suggest that a complex distribution of proteolytic labor maintains protein quality control in the inner membrane.

Graphical Abstract



Ribosomes that stall during protein synthesis are a common source of defective intracellular polypeptides.[1] In addition to producing an incomplete polypeptide, stalling depletes the pool of ribosomes available for translation and inhibits normal growth. In *Escherichia coli* and most eubacteria, the hybrid transfer-messenger RNA (tmRNA) system rescues stalled ribosomes and simultaneously appends a short sequence of C-terminal amino acids, called the ssrA tag, that targets the incomplete protein for degradation by ATP-fueled AAA+ proteases in the cytoplasm and by energy-independent proteases in the periplasm.[2,3] For example, ~10% of *E. coli* translation events appear to terminate with tmR-NA rescue and ssrA tagging,[1,4] but our understanding of how these ssrA-tagged aberrant proteins are

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Author Contributions

S.B.H. designed and performed all experiments. S.B.H. and R.T.S. contributed to data analysis and writing the manuscript.

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Methods and materials (PDF)

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degraded in different cellular compartments is incomplete, especially for membrane proteins.

E. coli contains five AAA+ proteases: ClpXP, ClpAP, FtsH, HslUV, and Lon, each consisting of a multimeric AAA+ ring that binds, unfolds, and translocates protein substrates into a degradation chamber formed by attached domains or separate peptidase subunits.[5] Cytoplasmic ssrA-tagged proteins are principally degraded by the ClpXP protease, with some help from ClpAP.[2,6,7] FtsH, which is anchored to the cytoplasmic face of the inner membrane, degrades soluble ssrA-tagged proteins *in vitro* and has been shown to degrade several integral membrane proteins *in vivo*.[8–11] Thus, it has been widely assumed that FtsH is also responsible for degrading ssrA-tagged membrane proteins.[12] Challenging this assumption, however, a recent study showed that ClpXP degrades ssrA-tagged AcrB, an *E. coli* inner-membrane protein.[13] Here, we investigate a different ssrA-tagged integral membrane protein and find that its degradation requires FtsH but not ClpXP or ClpAP *in vivo*. Thus, which AAA+ protease degrades which ssrA-tagged inner-membrane protein depends on factors that are currently poorly defined.

E. coli ProW is a multiple-pass inner-membrane protein.[14] For our studies, we used a truncated variant (ProW$_{1\text{-}182}$) with an N-terminal periplasmic domain, three transmembrane segments, and a C-terminus (with two added lysines) predicted to be cytosolic.[15] ProW$_{1\text{-}182}$ was fused to a FLAG tag and an ssrA tag (ProW$_{1\text{-}182}$-FLAG-ssrA; Fig. 1A) and cloned under control of an IPTG-inducible P_{trc} promoter. We also generated an otherwise identical construct in which the last two residues of the ssrA tag were replaced with aspartic acids (ssrA(DD)), as this mutant tag is not recognized by cellular proteases.[3] In ^{35}S pulse-chase experiments (Table 1 and Fig. 1B), ProW$_{1\text{-}182}$-FLAG-ssrA was degraded quickly ($t_{1/2}$ ~5 min), whereas ProW$_{1\text{-}182}$-FLAG-ssrA(DD) was not ($t_{1/2}$ > 60 min). The stability of ProW$_{1\text{-}182}$-FLAG-ssrA-(DD) allowed us to show that it purified with cellular membranes but was undetectable in the soluble cytosolic fraction in fractionation experiments (Fig. 1C). As expected from results using a very similar construct,[14] the activity of PhoA fusions to ProW$_{1\text{-}182}$-FLAG-ssrA confirmed that its C-terminus is cytoplasmic (Fig. 1D).

tmRNA-mediated addition of the ssrA tag *in vivo* can be induced using a non-stop mRNA that causes ribosome stalling at the 3′ end.[3] When ProW$_{1\text{-}182}$-FLAG was expressed from a gene with a strong transcriptional terminator and no stop codon (Fig. 2A), we observed ~80% ssrA-tagged ProW$_{1\text{-}182}$-FLAG (higher molecular-weight band) and ~20% untagged ProW$_{1\text{-}182}$-FLAG (lower molecular-weight band) at time zero in a pulse-chase experiment (Fig. 2B; left panel). Importantly, the tagged species was degraded rapidly ($t_{1/2}$ ~3 min) compared to the untagged species ($t_{1/2}$ ~60 min) (Table 1). As expected, the tagged species was not observed in a pulse-chase experiment performed in *ssrA* cells lacking tmRNA (Fig. 2B; right panel). Thus, whether ProW$_{1\text{-}182}$-FLAG contains a genetically encoded ssrA tag or acquires an ssrA tag as a consequence of ribosome stalling and tmRNA rescue, the tagged protein is rapidly degraded in *E. coli*.

To test the importance of different *E. coli* AAA+ proteases in intracellular degradation of ProW$_{1\text{-}182}$-FLAG-ssrA, we performed pulse-chase experiments (Fig. 3A) in cells lacking ClpP (the pep-tidase component of ClpXP and ClpAP), ClpX (the AAA+ un-foldase of

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ClpXP), ClpA (the AAA+ unfoldase of ClpAP), or FtsH (fused AAA+ unfoldase and peptidase). $ProW_{1-182}$-FLAG-ssrA was degraded at similar rates in a wild-type strain and otherwise isogenic strains lacking ClpP, ClpX, or ClpA. Thus, ClpXP and ClpAP do not contribute to intracellular degradation of $ProW_{1-182}$-FLAG-ssrA. Strikingly, however, deletion of FtsH resulted in much slower degradation of $ProW_{1-182}$-FLAG-ssrA ($t_{1/2} > 60$ min) compared to an otherwise isogenic wild-type strain ($t_{1/2}$ ~5 min). We also used the non-stop stalling construct to induce ssrA tagging of $ProW_{1-182}$-FLAG in cells without FtsH (Fig. 3B). The ssrA-tagged species was observed in the wild-type and *FtsH::Kan* strains but persisted only in the latter strain ($t_{1/2}$ 60 min). Taken together, these results show that rapid degradation of the ssrA-tagged $ProW_{1-182}$ inner-membrane protein depends upon FtsH.

Insertion of proteins into the inner membrane occurs rapidly, on time scales comparable to their rates of biosynthesis.[16] For $ProW_{1-182}$-FLAG-ssrA, synthesis should take ~15 s. To test if FtsH degrades $ProW_{1-182}$-FLAG-ssrA after membrane insertion, we transformed *E. coli ftsH::Kan* with one plasmid encoding $ProW_{1-182}$-FLAG-ssrA under IPTG control and a second plasmid encoding myc-FtsH under anhydrotetracyline (ATc) control. After briefly inducing synthesis of $ProW_{1-182}$-FLAG-ssrA from P_{trc} with IPTG, we added phenyl-D-galactopyranoside (pGAL) to repress further synthesis. Two min after pGAL addition, one aliquot of cells was treated with ATc to induce myc-FtsH expression and a control aliquot was mock treated. As shown in the upper panel of Fig. 3C, when FtsH synthesis was not induced, the level of intracellular $ProW_{1-182}$-FLAG-ssrA remained relatively constant from the time of pGAL addition until ~40 min. By contrast, $ProW_{1-182}$-FLAG-ssrA was completely degraded after 40 min in cells in which myc-FtsH synthesis was induced. We conclude that FtsH-mediated degradation of $ProW_{1-182}$-FLAG-ssrA can occur after this protein has been inserted into the inner membrane.

To study degradation *in vitro*, we purified His_6-tagged variants of FtsH and ClpXP and first verified that they efficiently degraded Arc-ssrA (Fig. 4A, right lanes), a soluble model substrate previously shown to be degraded by both proteases.[9,17] Next, we tested FtsH and ClpXP degradation of purified HA-$ProW_{1-182}$-His_6-ssrA. We used this substrate because it was easier to purify than $ProW_{1-182}$-FLAG-ssrA. FtsH and ClpXP both degraded HA-$ProW_{1-182}$-His_6-ssrA, but roughly 10X more FtsH than ClpXP was required to achieve a comparable rate of degradation (Fig. 4).

Degradation of ssrA-tagged $ProW_{1-182}$ *in vivo* requires FtsH but it is not affected by deletion of ClpXP or ClpAP. Degradation of ssrA-tagged $ProW_{1-182}$ *in vitro* was observed with both FtsH and ClpXP, but ClpXP degradation was ~10-fold more efficient. Several factors could be responsible for these differences. For example, the detergent-solubilized environment of the substrate *in vitro* might interfere with FtsH degradation, FtsH and the substrate might need to be present in the same membrane for efficient recognition and degradation, and/or an additional cellular component or components required for efficient FtsH degradation might be missing in our reconstituted assay. The fact that ClpXP degrades ssrA-tagged $ProW_{1-182}$ *in vitro* but not *in vivo* is likely to be a consequence of the substrate being embedded in the inner-membrane *in vivo*, which could reduce accessibility of the ssrA tag and/or create an energetic barrier that is too high for ClpX to pull ssrA-tagged $ProW_{1-182}$ out of the

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membrane. Further studies will be required to distinguish between these mechanistic possibilities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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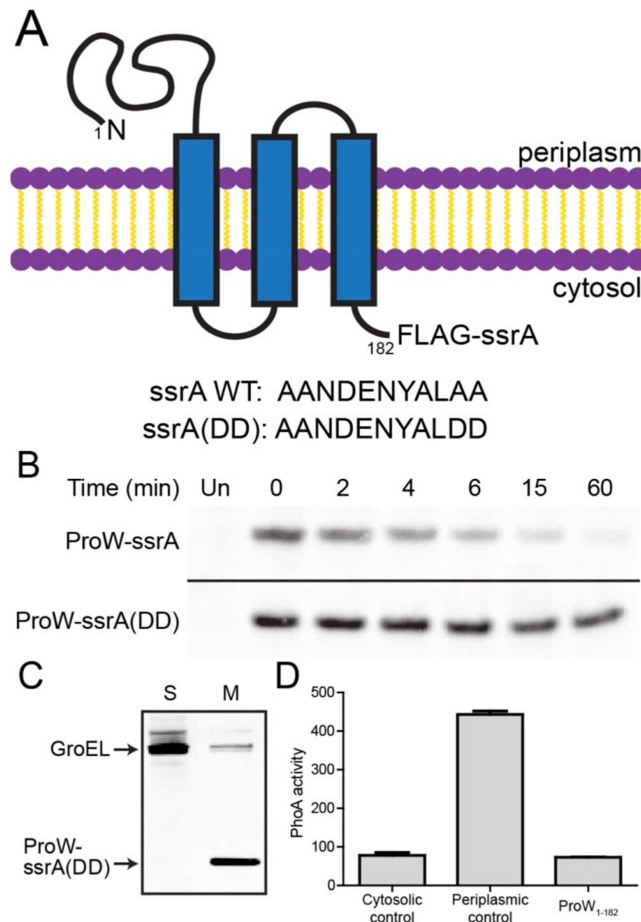

Figure 1.

An ssrA-tagged model membrane protein is degraded in *E. coli.* (A) Topology of $ProW_{1\text{-}182}$-FLAG-ssrA (top) and amino-acid sequences of wild-type and mutant ssrA tags (bottom). (B) Expression of $ProW_{1\text{-}182}$-FLAG-ssrA or $ProW_{1\text{-}182}$-FLAG-ssrA(DD) in *E. coli* X90 was induced, pulsed with ^{35}S-labeled methionine and cysteine, and chased with unlabeled amino acids. Samples taken at different times were immunoprecipitated and analyzed by SDS-PAGE and autoradiography. "Un" indicates an uninduced control sample. (C) *E. coli* cells expressing $ProW_{1\text{-}182}$-FLAG-ssrA(DD) were lysed and fractionated into soluble (S) and membrane (M) fractions, which were analyzed by SDS-PAGE and immunoblotted using antibodies against GroEL (a cytosolic protein) and the FLAG tag. (D) Activity (in Miller units) of C-terminal FLAG-ssrA-PhoA fusions of a cytosolic protein (λ repressor), a periplasmic protein (cytochrome b_{562}), and $ProW_{1\text{-}182}$.

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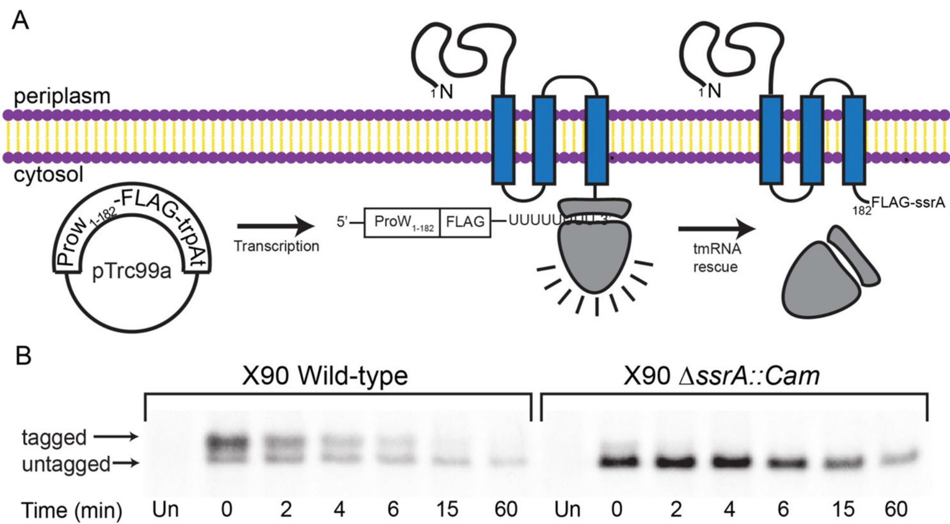

Figure 2.

tmRNA-mediated tagging of $ProW_{1\text{-}182}$ results in rapid degradation. (A) A non-stop mRNA encoding $ProW_{1\text{-}182}$-FLAG results in ribosomal stalling and ssrA tagging. (B) A plasmid encoding $ProW_{1\text{-}182}$-FLAG-trpAt was transformed into wild-type *E. coli* X90 or an otherwise isogenic *ssrA::Cam* strain, and the half-life of the tagged and untagged proteins was measured by pulse-chase experiments as described in Fig. 1B.

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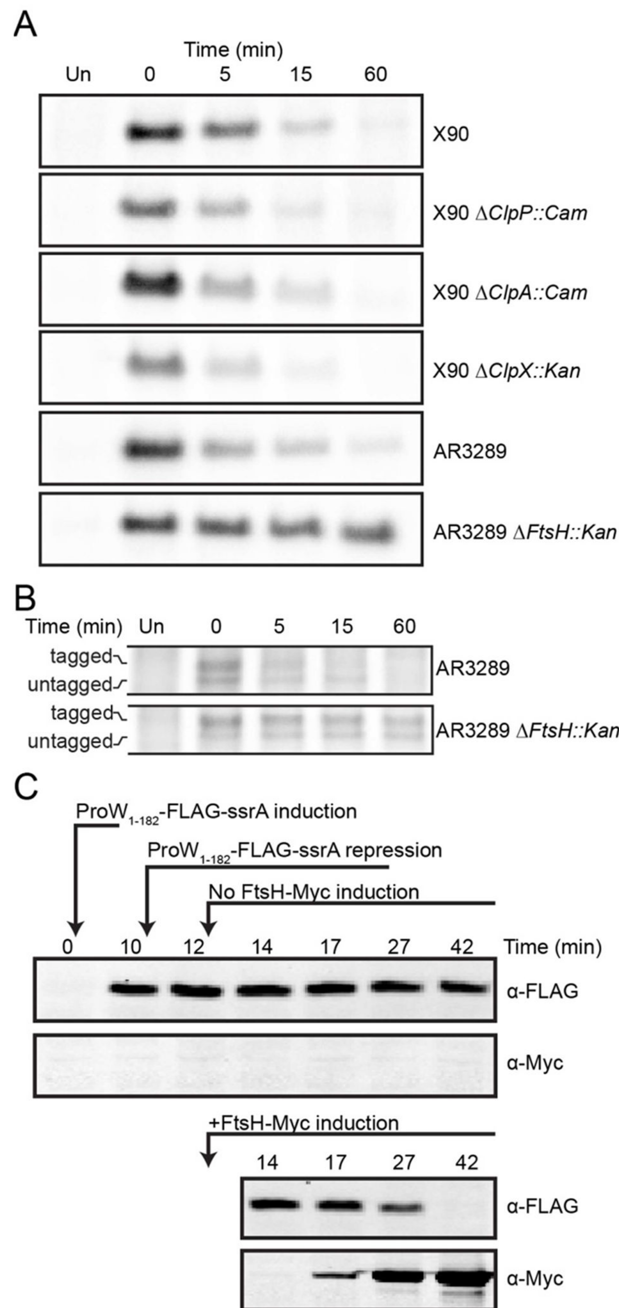

Figure 3.

FtsH degrades ProW$_{1-182}$-FLAG-ssrA. (A) Pulse-chase experiments demonstrate that ProW$_{1-182}$-FLAG-ssrA has a longer half-life in cells lacking FtsH compared to wild-type cells or those lacking ClpP, ClpX, or ClpA (see Fig. 1B legend for experimental details). (B) Auto-radiograms of whole-cell lysates from pulse-chase experiments show that endogenously ssrA-tagged ProW$_{1-182}$-FLAG-trpAt is also stabilized in a strain lacking FtsH. (C) Transient induction of plasmid-encoded ProW$_{1-182}$-FLAG-ssrA in *E. coli* AR3289 *ftsH::Kan* resulted in little degradation (top panels) unless expression of plasmid-encoded Myc-tagged FtsH was subsequently induced (bottom panels). In all panels, ProW$_{1-182}$-

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FLAG-ssrA expression was induced with IPTG for 10 min, and then pGAL was added to repress further expression. In the bottom panels, expression of Myc-tagged FtsH was induced by addition of ATc two min after addition of pGAL. Samples were separated by SDS-PAGE and immunoblotted with anti-FLAG and anti-Myc tag antibodies.

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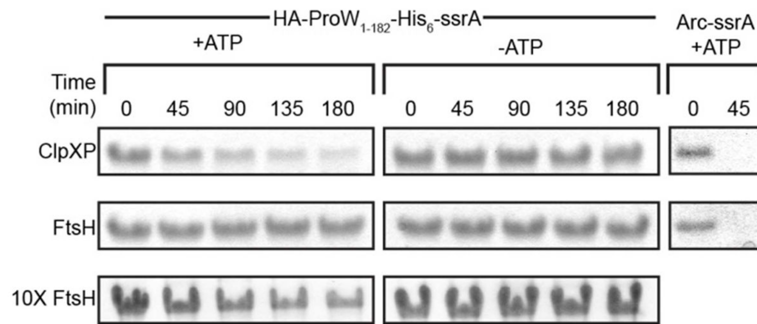

Figure 4.

In vitro degradation of a model membrane protein. Purified HA-ProW$_{1\text{-}182}$-His$_6$-ssrA was degraded by purified ClpXP (top panel) or FtsH (middle and bottom panels) in ATP-dependent reactions. Arc-ssrA was also degraded by both proteases. Reactions were initiated by addition of 4 mM ATP (with regeneration system), quenched at different times, and then analyzed by SDS-PAGE and staining with Coomassie Blue.

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Table 1

Half-lives of ProW constructs determined from two independent experiments. n.d. – not determined.

	Half-life (min)	
	X90	**X90** ***ssrA::Cam***
$ProW_{1\text{-}182}$-FLAG-ssrA	7, 4	n.d.
$ProW_{1\text{-}182}$-FLAG-ssrA(DD)	>60, >60	n.d.
$ProW_{1\text{-}182}$-FLAG-trpAt	3, 2	~60, ~60