

# Nascent Lep inserts into the *Escherichia coli* inner membrane in the vicinity of YidC, SecY and SecA

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**Abstract** Targeting and assembly of the *Escherichia coli* inner membrane protein leader peptidase (Lep) was studied using a homologous in vitro targeting/translocation assay. Assembly of full-length Lep was efficient in the co-translational presence of membrane vesicles and hardly occurred when membranes were added post-translationally. This is consistent with the signal recognition particle-dependent targeting of Lep. Crosslinking experiments showed that the hydrophilic region P1 of nascent membrane-inserted Lep 100-mer was in the vicinity of SecA and SecY, whereas the first transmembrane domain H1 was in the vicinity of YidC. These results suggested that YidC, together with the Sec translocase, functions in the assembly of Lep. YidC might be a more generic component in the assembly of inner membrane proteins. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Translocon; Membrane protein; Leader peptidase; YidC; *Escherichia coli*

## 1. Introduction

Leader peptidase (Lep) is an *Escherichia coli* inner membrane protein that spans the membrane twice with both the short N-terminus and its large C-terminal domain (P2) facing the periplasmic side. The protein has been extensively used as a model for in vivo inner membrane insertion [1–4]. Translocation of the P2 loop requires the translocase components SecY, SecE and SecA [1,4,5] but translocation of the N-terminal tail does not [1]. The mechanism of translocation of the P2 domain is dependent on its length [2]. Periplasmic loops smaller than 60 amino acid residues pass the membrane in an apparently spontaneous process, while translocation of longer loops, like P2 of Lep, requires the action of the Sec translocase.

Originally, the Sec machinery was found to be essential for translocation of periplasmic and outer membrane proteins across the inner membrane (reviewed in [6]). Unlike Lep, these proteins are synthesized with an N-terminal cleavable signal sequence and are kept in a translocation-competent state by SecB. The SecB–preprotein complex is targeted to the trans-

locase in a post-translational or late co-translational fashion by virtue of its high affinity for the membrane-embedded translocase. SecB is released from the preprotein as the ATPase SecA mediates translocation through the SecYEG translocon by ATP-driven cycles of insertion and de-insertion.

Lep does not need SecB for its targeting [4]. Like many other inner membrane proteins [7], Lep requires the signal recognition particle (SRP) for efficient routing to the inner membrane [3]. The SRP consists of a small 4.5S RNA and a 48 kDa protein (designated P48 or ffh) that interacts with targeting sequences of nascent proteins [8–10]. The SRP supports co-translational targeting and membrane insertion together with its receptor FtsY in a GTP-controlled process that resembles the mechanism of targeting of proteins to the eukaryotic endoplasmic reticulum (reviewed in [11]). Consistent with a conserved mechanism of membrane insertion Lep inserts in mammalian microsomes in a compulsory co-translational and SRP-dependent manner [12]. Photo-crosslinking studies demonstrated that Lep interacts transiently with the SecY homologue Sec61 $\alpha$  in the membrane of the endoplasmic reticulum before engaging the membrane lipids [13].

In the present work, we used a homologous in vitro translation/translocation system to confirm the preferred co-translational mode of membrane assembly of Lep into inverted *E. coli* inner membrane vesicles (IMVs). In addition, we have analyzed the molecular environment of nascent (ribosome-associated) Lep 100-mer (100Lep) in the IMVs after SRP-mediated targeting by using bifunctional chemical and photoreactive crosslinking reagents. We found the first transmembrane domain (H1) to be close to YidC, a recently discovered translocase component that is homologous to mitochondrial Oxa1p [14]. The hydrophilic domain preceding H1 (P1) is adjacent to SecY and SecA. A model for the topology of membrane-inserted nascent Lep is discussed.

## 2. Materials and methods

### 2.1. Enzymes and materials

Restriction enzymes and Taq polymerase were from Roche Molecular Biochemicals GmbH (Mannheim, Germany). T4 RNA ligase was from Epicenter Technologies (Madison, WI, USA). Megashortscript T7 transcription kit was from Ambion Inc. (Austin, TX, USA). [<sup>35</sup>S]Methionine and protein A-Sepharose were from Amersham International (Buckinghamshire, UK). Disuccinimidyl suberate (DSS) was from Pierce (Rockford, IL, USA). All other chemicals were supplied by Sigma Chemical Co. (St. Louis, MO, USA).

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## 2.2. Strains and plasmid constructs

Strain MC4100 was used to obtain translation lysates and IMVs (both prepared essentially as described in [15]). Strain MRE600 was used to prepare translation lysate for suppression of TAG stop codons in the presence of 4-(3-trifluoromethyl-3-diaziriny)phenylalanyl-tRNA<sup>sup</sup> ((Tmd)Phe-tRNA<sup>sup</sup>) [16], except that lysates were cleared from membranes at 135 000 × *g* according to [15]. Strain Top10F' was used for routine maintenance of plasmid constructs [9]. pBSK-PhoE-WT was a gift of Jan Tommassen and was used for the preparation of full-length PhoE mRNA. pGEM3-Lep was used for the preparation of full-length Lep mRNA. To construct pGEM3-Lep, the Lep coding sequence was cloned from pGEM1-Lep [12] into pET21d (Novagen, Madison, WI, USA) using *Nco*I and *Sac*I. Subsequently, the *Xba*I–*Sal*I fragment of pET21d-Lep was cloned into pGEM3. pC4Meth93-Lep [9] was used to prepare truncated Lep mRNA and as a template in a two-step PCR procedure to introduce TAG stop codons at positions 3, 10, 15 and 47. The nucleotide sequences of the mutant genes were confirmed by DNA sequencing.

## 2.3. In vitro transcription, translation, translocation and crosslinking

To prepare full-length and truncated mRNA, the Lep and PhoE derivative plasmids were linearized with *Hind*III or *Cla*I and transcribed using T7 polymerase as described by the manufacturer (Ambion Inc., Austin, TX, USA). Full-length transcripts were used in in vitro translation/translocation reactions containing [<sup>35</sup>S]methionine essentially as described [17]. In a co-translational reaction, IMVs were added at the start of translation, which continued for 20 min at 37°C and was terminated by addition of chloramphenicol (30 µg/ml for 5 min at 0°C). In a post-translational reaction, IMVs were added after the translation reaction had been terminated with chloramphenicol (see above) and the translocation reaction was continued for 5 min at 37°C. The samples were treated with proteinase K (200 µg/ml) with or without 1% Triton X-100 for 30 min at 0°C. Proteolysis was stopped by the addition of trichloroacetic acid (TCA) to 20%. The samples were analyzed by SDS-PAGE and radiolabeled bands were quantified. The quantification was corrected for the number of methionines present in the labeled protein (or fragment). Truncated Lep transcripts were used in co-translational in vitro translation/targeting reactions as described [17]. Bifunctional crosslinking was induced with 2 mM DSS for 10 min at 25°C and quenched at 0°C by adding 1/10 volume of quench buffer (1 M glycine, 100 mM NaHCO<sub>3</sub>, pH 8.5). To separate integral membrane from soluble and peripheral crosslinked complexes, samples were treated with 0.18 M Na<sub>2</sub>CO<sub>3</sub> (pH 11.3) for 15 min at 0°C [17]. For photo-crosslinking, (Tmd)Phe was site-specifically incorporated into Lep nascent chains by suppression of UAG stop codons using (Tmd)Phe<sup>sup</sup> in an in vitro translation/targeting system as described [14]. Photo-crosslinking was carried out as described [14]. After crosslinking, membrane fractions containing integral membrane proteins were collected by Na<sub>2</sub>CO<sub>3</sub> extraction (see above) and resuspended in RN buffer (100 mM KOAc, 5 mM Mg(OAc)<sub>2</sub>, 50 mM HEPES-KOH, pH 7.9). Both pellet and supernatant fractions were either TCA-precipitated or immunoprecipitated as described [18]. The material used for immunoprecipitation was 10-fold (Fig. 2) or two-fold (Fig. 3) the amount used for TCA precipitation.

## 2.4. Antisera

Agrisera (Umeå, Sweden) raised the YidC polyclonal antiserum in rabbit against a peptide that consisted of the 17 C-terminal amino acids of YidC. The antiserum against SecY was a gift of A. Driessen.

## 2.5. Sample analysis and quantification

All samples were analyzed on 13% or 15% SDS-polyacrylamide gels. Radiolabeled proteins were visualized by phosphor imaging using Molecular Dynamics PhosphorImager 473 and quantified using the Imagequant quantification software from Molecular Dynamics.

## 3. Results

Using an in vitro translation/targeting system we have shown previously that nascent 100Lep interacts with the SRP and associates with IMVs [8,9]. These observations suggest a co-translational mode of membrane insertion. To study the mechanism of biogenesis of Lep in more detail, full-length

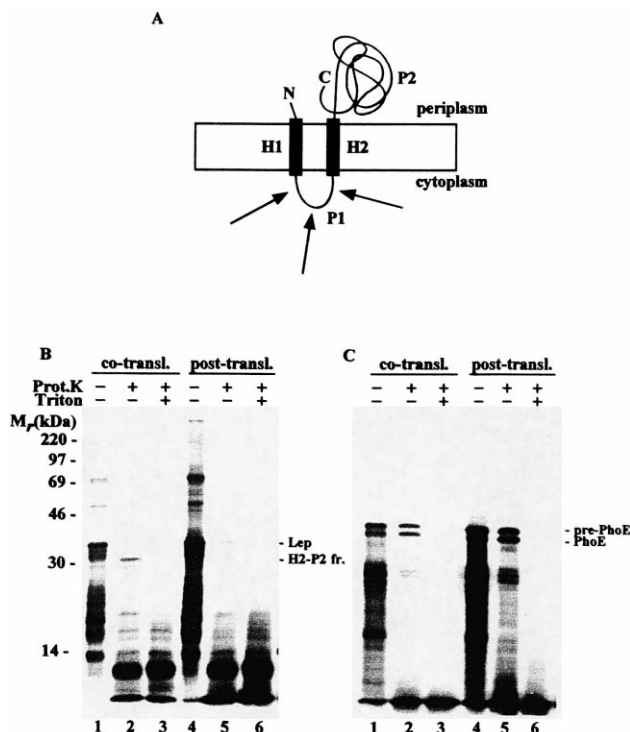


Fig. 1. Membrane assembly of Lep occurs co-translationally. A: Orientation of Lep in the cytoplasmic membrane. The H1, H2, P1 and P2 regions are indicated. The arrows indicate the proteinase K-sensitive P1 domain of Lep in IMVs. B, C: Full-length Lep (B) and PhoE (C) were synthesized in the presence or absence of IMVs (as indicated) for 20 min after which translation was stopped with chloramphenicol. After 5 min IMVs were added to the latter samples and the incubation was continued for 5 min. Aliquots were treated with proteinase K and Triton X-100 as indicated. Full-length Lep and the protected H2-P2 fragment are indicated as well as the precursor and mature forms of PhoE (pre-PhoE and PhoE, respectively). The identity of these products was verified by immunoprecipitation (not shown). Co-translational addition of IMVs inhibits the efficiency of translation as has been documented before.

Lep was synthesized in the same translation system. IMVs were either added from the start (co-translationally) or after termination of translation (post-translationally) and the translocation of the P2 loop was monitored in a protease protection assay (see Fig. 1A). As shown in Fig. 1B, part (quantified as 12%) of Lep was assembled correctly upon co-translational addition of IMVs as judged by protection of the H2-P2 region against proteinase K in the absence of Triton X-100 (Fig. 1B, lanes 1–3). As expected, assembly was not affected when the translation lysate was derived from a SecB knock-out strain (N. Harms, unpublished data). In contrast, post-translational addition of IMVs hardly (quantified as <1%) resulted in the assembly of Lep (Fig. 1B, lanes 4 and 5).

As a control, PhoE import was monitored in the same assay (Fig. 1C). PhoE is a SecB-dependent outer membrane porin that is able to translocate post-translationally [15]. PhoE import was hardly affected by the timing of addition of IMVs: co-translational addition resulted in 23% translocation (Fig. 1C, lanes 1 and 2) whereas in the post-translational setting, 19% translocation was observed (Fig. 1C, lanes 4 and 5). In both cases, assembly was significantly decreased when SecB was not present in the translation lysate indicative of SecB-mediated targeting independent of the timing of the addition

of IMVs (data not shown). Taken together, these results suggest that membrane assembly of Lep occurs almost exclusively co-translationally in our experimental assay. Possibly, Lep synthesized in the absence of IMVs rapidly acquires a conformation that is not compatible with proper membrane insertion and assembly.

Previously, we have used the *in vitro* translation/targeting system to analyze the route of targeting and membrane insertion of the bitopic type II (N-in, C-out) inner membrane protein FtsQ by studying the interactions of nascent FtsQ both in the cytosol and in the membrane [14,17]. Here, we used the same approach to study the membrane insertion of nascent 100Lep. At this nascent chain length the first transmembrane domain H1 (residues 4–22) that acquires a type I topology (N-out, C-in) in the membrane is exposed outside the ribosome as is the hydrophilic P1 loop. Most of the second transmembrane region H2, on the other hand, is expected to be in the ribosome that covers 35–40 residues. 100Lep was synthesized in the presence of IMVs to allow targeting [17] and the samples were treated with the homobifunctional crosslinking reagent DSS (Fig. 2, lanes 1 and 2). After crosslinking, the samples were extracted with alkaline sodium carbonate buffer to separate integral membrane (Fig. 2, lane 3) from peripheral and soluble crosslinked complexes (Fig. 2, lane 4). In the carbonate pellet crosslinking adducts of ~120 kDa, ~60 kDa and ~42 kDa were present, which could be immunoprecipitated with anti-SecA, anti-P48 and anti-SecY, respectively (Fig. 2,

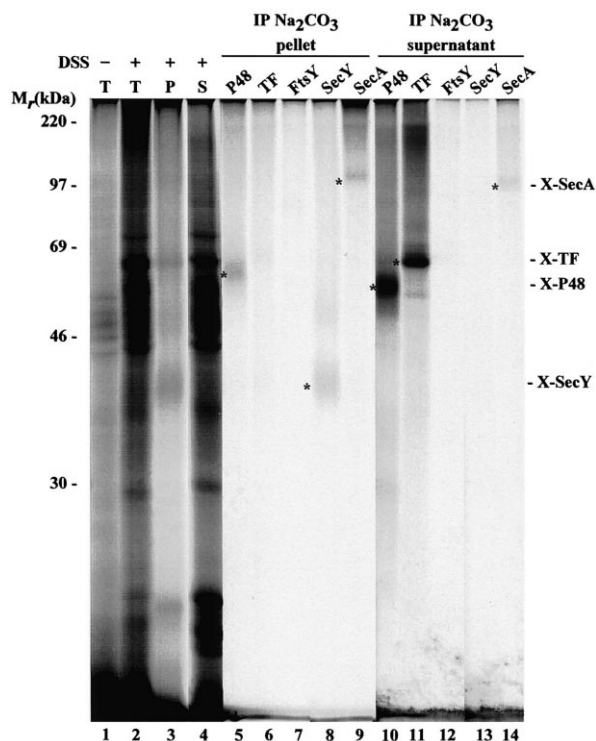


Fig. 2. 100Lep interacts with the translocon components SecA and SecY. 100Lep was synthesized in the presence of IMVs and treated with DSS. The samples were quenched and aliquots were TCA-precipitated (T, lanes 1 and 2). Soluble and peripheral crosslinking complexes were extracted from the membranes with  $\text{Na}_2\text{CO}_3$  as described in Section 2. Both pellet (P, lane 3) and supernatant (S, lane 4) fractions were examined by immunoprecipitation (IP, lanes 5–14) for the identification of crosslinking adducts using sera specific for the indicated proteins.

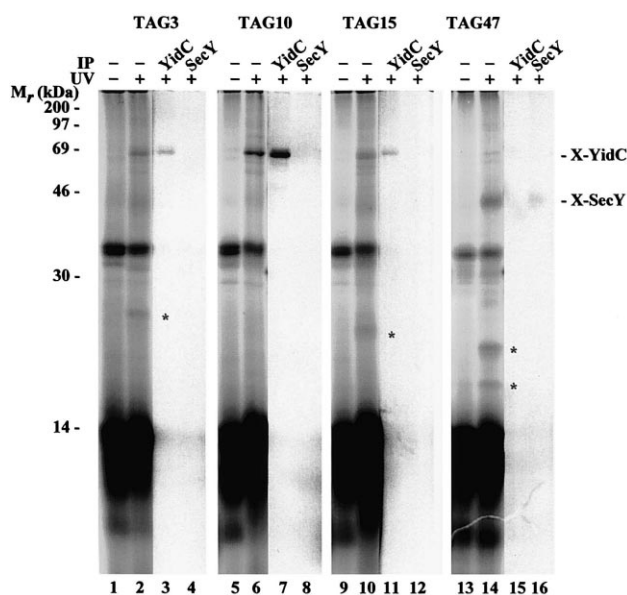


Fig. 3. The first transmembrane domain of membrane-inserted 100Lep interacts with YidC, while the P1 loop is close to SecY. Translation of 100Lep was carried out in the presence of IMVs and in the presence of (Tmd)Phe-tRNA<sup>sup</sup>. Upon translation samples were UV-irradiated or kept in the dark as indicated for 10 min at 4°C and extracted with  $\text{Na}_2\text{CO}_3$  to remove soluble and membrane peripheral proteins. UV-irradiated samples were immunoprecipitated using antisera against YidC and SecY as indicated. Smaller crosslinking adducts (indicated by asterisks) were not identified.

lanes 5, 8 and 9). In the carbonate supernatant, a smear of crosslinking products was observed (Fig. 2, lane 4) that contains adducts to P48, trigger factor and SecA as revealed by immunoprecipitation (Fig. 2, lanes 10, 11 and 14). Crosslinking to FtsY (which migrates close to SecA in SDS-PAGE) was not detected (Fig. 2, lanes 7 and 12). Qualitatively, the crosslinking patterns are very similar to those obtained with nascent 108FtsQ [17]. These results suggest that 100Lep is targeted by the SRP to the membrane and forms a translocation intermediate close to the translocase components SecA and SecY.

The crosslinking reagent DSS is specific for lysine residues and thus biased to detect interactions of proteins with the hydrophilic P1 loop. To analyze interactions with H1 and the translocated N-terminus more directly, an alternative, site-specific, photo-crosslinking approach was employed [19]. Single stop (TAG) codons were introduced in 100Lep at position 3 in the N-terminal translocated region, at positions 10 and 15 in the H1 domain and at position 47 in the P1 domain. The TAG codons were suppressed during *in vitro* synthesis by addition of (Tmd)Phe-tRNA<sup>sup</sup>, which carries a highly reactive carbene-generating photoreactive probe that is completely non-selective in its reaction upon UV irradiation [19].

In the absence of (Tmd)Phe-tRNA<sup>sup</sup> 100Lep was not produced (not shown). Addition of (Tmd)Phe-tRNA<sup>sup</sup> to the translation reaction suppressed the TAG mutations, resulting in significant amounts of nascent 100Lep, which was targeted efficiently and became membrane-inserted as judged by its resistance to sodium carbonate extraction (not shown). UV irradiation of TAG3, TAG10 and TAG15 nascent chains induced the formation of a ~68 kDa adduct in the membrane fraction that represents crosslinking to YidC as judged by immunoprecipitation (Fig. 3, lanes 1–3, 5–7 and 9–11). Cross-

linking to YidC was strongest at position 10, which is located in the middle of the H1 domain. UV irradiation of TAG47 nascent chains resulted in the appearance of a crosslinking adduct of ~44 kDa as compared to the non-irradiated sample (Fig. 3, lanes 13 and 14). The crosslinking partner was identified as SecY by immunoprecipitation (Fig. 3, lane 16). In contrast, no significant crosslinking to YidC was observed (Fig. 3, lanes 14 and 15). The ~44 kDa crosslinking adduct was hardly visible in the TAG3, 10 and 15 samples and could not be immunoprecipitated with anti-SecY to a detectable level (Fig. 3, lanes 2, 4, 6, 8, 10 and 12). Several other crosslinking adducts were detected in the ~14–30 kDa range (indicated by asterisks) that remain to be identified. Together, the data suggest that the N-terminus and H1 region of membrane-inserted 100Lep are in close proximity to YidC while the hydrophilic P1 domain is close to SecY.

#### 4. Discussion

Using a homologous *in vitro* targeting assay we show here that nascent Lep is able to insert into the *E. coli* inner membrane at a translocation site that contains SecA, SecY and YidC.

*In vitro* translocation of the P2 domain of full-length Lep could only be detected at a significant level when IMVs were present during translation suggesting compulsory co-translational membrane integration consistent with its SRP-dependent targeting *in vivo* [3,9]. Even in the presence of IMVs the efficiency of translocation was relatively low compared to the post-translational translocation of pre-PhoE. Possibly, in the dilute *in vitro* system, targeting is relatively slow which could result in a translocation-incompetent conformation of Lep. In a previous *in vitro* integration study small but significant amounts of Lep were inserted post-translationally in an ATP-independent mechanism which may be due to subtle differences in the experimental systems [20].

Nascent 100Lep that exposes H1 and the P1 loop outside the ribosome has previously been shown to associate with IMVs [17]. Based on the bifunctional and photo-crosslinking data presented, we propose a model for the topology and interactions of this translocation intermediate in the membrane (Fig. 4). Strikingly, H1, which is a reverse signal anchor (N-out, C-in), is in close contact with YidC, the homologue of mitochondrial Oxa1p. Oxa1p was shown to play an, as yet undefined, role in the membrane assembly of proteins that carry a large translocated N-tail in the mitochondrial inner membrane [21–23]. Recently, we showed that the signal anchor sequence of membrane-inserted nascent 108FtsQ (N-in, C-out) is also adjacent to YidC [14]. We also showed that YidC is associated with the Sec translocase suggesting an intimate structural and functional connection [14]. The fact that YidC is found in contact with a 'normal' and reverse signal anchor sequence of two inner membrane proteins of different complexity makes it likely that YidC is a generic component involved in the biogenesis of different types of inner membrane proteins. Apparently, the same molecular machinery is involved in the initial steps of membrane integration of proteins with different topology.

At the analyzed nascent chain length the hydrophilic P1 loop which contains six exposed lysine residues was cross-linked to SecA and SecY using DSS. More in particular, position 47 was specifically photo-crosslinked to SecY. What

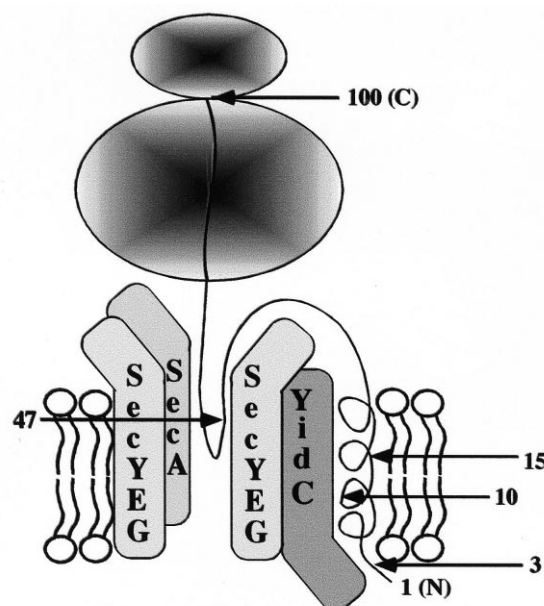


Fig. 4. Model for the molecular environment of membrane-inserted nascent 100Lep.

is the order of the interactions with YidC and SecY during membrane insertion? According to existing models, the primary insertion of H1 would take place at SecY before lateral diffusion into the lipid bilayer [24]. The latter process could be assisted by YidC. However, a more direct role of YidC in the reception of Lep H1 cannot be excluded. Lep carries a small translocated N-tail reminiscent of N-tail proteins in mitochondria that require Oxa1p for assembly in the inner membrane that is devoid of Sec translocase [25]. Interestingly, a role distinct from the Sec translocase was also recently proposed for the chloroplast homologue of Oxa1p, ALB3, which appeared to be required for the assembly of the SRP substrate LHCP into the thylakoid membrane [26]. In support of a role of YidC in the reception of Lep H1 is the observation that H1 inserts independently of SecA and SecY *in vivo*. It should be noted, however, that this conclusion was based on the translocation of the N-terminus (fused to an epitope) in SecA/Y conditional strains in which it is difficult to completely eliminate the Sec function.

Future studies will concentrate on a more detailed dissection of consecutive interactions of nascent Lep in the membrane using varied lengths of nascent chains that should provide snapshots of the membrane integration process.

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