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The conserved extension of the Hbp autotransporter signal peptide does not determine targeting pathway specificity

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Abstract

Autotransporters (ATs) of Gram-negative bacteria are often produced with an unusual signal peptide that carries a conserved N-terminal extension. Using combined *in vitro* and *in vivo* approaches we show that the extension of the AT hemoglobin protease (Hbp) does not affect targeting of Hbp via the SRP-pathway, suggesting that the extension is not involved in targeting pathway selection.

Keywords: Type V secretion; Autotransporter; Signal peptide; SRP; SecB; Protein targeting; *Escherichia coli*

Many virulence factors of Gram-negative bacteria are secreted into the extracellular milieu via the autotransporter (AT) secretion pathway. Upon synthesis, ATs comprise three functional domains: (i) the N-terminal signal peptide that mediates targeting to and initiation of transfer through the Sec-translocon, the major protein conducting channel in the inner membrane, (ii) the secreted passenger domain that carries the actual effector function of the AT, and (iii) the β-domain at the C-terminus that mediates translocation of the passenger domain across the outer membrane [1]. Many steps of the AT secretion mechanism are still under debate, including the targeting of ATs to in the inner membrane.

A subset of autotransporters, including all members of the subfamily of serine protease autotransporters of enterobacteriaceae (SPATEs), is synthesized with an unusually long cleavable signal peptide. The C-terminal half of these peptides resembles a classical signal peptide and is not particularly conserved. In contrast, the N-terminal half (approximately 25 amino acids) is remarkably conserved [2,3]. The precise function of this extension has remained controversial.

Most secretory proteins synthesized with cleavable signal peptides reach the Sec-translocon via the post-translational SecB targeting pathway [4]. Remarkably, the signal recognition particle (SRP) that mediates co-translational targeting of primarily inner membrane proteins (IMPs) [5], was shown to play a role in targeting of the SPATE hemoglobin protease (Hbp) of human pathogenic *Escherichia coli* [6]. SecB appeared dispensable for targeting of Hbp but could largely compensate for a lack of SRP, suggesting a facultative use of either the SRP- or SecB-pathway [6].

SRP preferentially interacts with relatively hydrophobic targeting sequences such as those present in IMPs [7,8]. However, the signal peptides of Hbp and other ATs are not particularly hydrophobic [2]. It was speculated that the conserved extension of AT signal peptides supports binding of the SRP [3,6]. Suggested alternative roles for the extension include regulation of the kinetics of inner membrane translocation of ATs and promotion of post-translational targeting of ATs to the Sec-translocon via a novel mechanism [9–12]. Here, we investigated the role of the conserved AT signal peptide extension in the targeting

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of Hbp using combined *in vitro* (crosslinking) and *in vivo* (pulse-chase) approaches.

Materials and methods

Reagents and sera. Restriction enzymes, expand long template PCR system and T4-DNA ligase were purchased from Roche Applied Science. [^{35}S]Methionine and Protein A Sepharose were from Amersham. Other chemicals were supplied by Sigma–Aldrich. Reconstituted SRP was a gift from Dr. I. Sinning (Heidelberg University, Germany). Antisera were from our collection.

Strains and plasmids. Escherichia coli K-12 strains used were TOP10F' (Invitrogen) and HDB52 [7]. For *in vitro* crosslinking experiments and *in vivo* pulse-chase experiments, Hbp constructs were cloned into plasmids pC4Meth and pEH3, respectively, as described previously [6,13]. Details on plasmid construction can be found online as Supplementary material.

In vitro transcription, translation, and crosslinking. Preparation of truncated mRNA, *in vitro* translation, chemical- or photo-crosslinking and sample processing were performed as described previously [6,14].

Pulse-chase analysis. Pulse-chase experiments were carried out at 37 °C. Cells were induced with 1 mM of IPTG, pulse labeled with 10 μCi/ml [^{35}S]methionine and chased with 2 mM cold methionine. Culture samples were added to one volume of ice-cold M9 medium to stop the chase. Cells and spent medium were separated by centrifugation. Medium fractions were TCA-precipitated whereas cells were subjected to immunoprecipitation using Hbp(J40) antiserum as described [6].

Results and discussion

The extension is not required for inner membrane targeting and secretion of Hbp

To investigate the role of the conserved extension of the Hbp signal peptide, we constructed an Hbp-mutant, Hbp[ΔExt], that lacks the first 23 amino acid residues following the initiation Met[1] of the Hbp signal peptide corresponding to the conserved extension (Fig. 1). Hbp[ΔExt] and wild-type Hbp were cloned under *lac* promoter control. To analyze the effect of deletion of the extension on targeting and export, the processing kinetics of Hbp and Hbp[ΔExt] were analyzed in *E. coli* strain TOP10F' in a pulse-chase experiment. Furthermore, spent medium of the cultures was analyzed to monitor the influence on secretion (Fig. 2). As shown previously [6], the processing kinetics of wild-type Hbp are characterized by relatively fast processing of the signal peptide (conversion of 'preproHbp' to 'proHbp'), somewhat slower processing of the C-terminal β-domain (conversion of 'proHbp' to 'pass') and much slower release of the passenger from the cells into the medium (Fig. 2, lanes 1–5). The kinetics of processing and release of Hbp[ΔExt] appeared essentially identical to those of wild-type Hbp (Fig 2, lanes 6–10). This indicates that the conserved extension is not required for targeting and secretion of Hbp. Consistently, it was recently shown that removal of the signal peptide extension of the SPATE EspP does not affect translocation of this protein across the inner membrane [12]. However, translocation of EspP across the outer membrane was impaired in the absence of the extension. Notably, this effect was reported to be influenced by both growth conditions and the level of EspP synthesis [12], which might account for the discrepancy with our results.

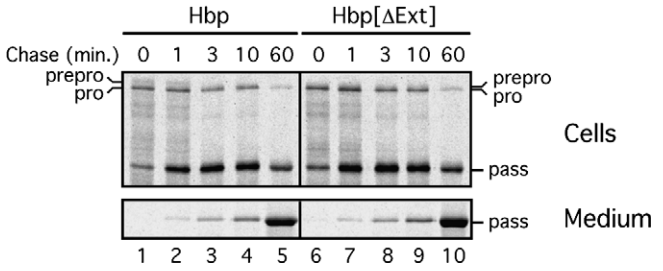

Fig. 2. The conserved signal peptide extension is not required for biogenesis of Hbp. Processing and secretion of Hbp and Hbp[ΔExt] in *E. coli* TOP10F' was analyzed in a pulse-chase experiment, essentially as described previously [14]. Cells were grown in M9 medium containing a methionine and cysteine-free amino acid mixture and 0.4% glucose. Cells were induced with IPTG for 6 min, labeled with [^{35}S]methionine for 1 min and chased for the times indicated. Hbp was immunoprecipitated from cell fractions or TCA-precipitated from medium fractions. Samples were analyzed by SDS–PAGE and phosphorimaging. Prepro-, pro-, and passenger-forms of Hbp and Hbp[ΔExt] are indicated.

The extension is not critical for recognition by SRP

To analyze whether the extension influences the mode of targeting to the inner membrane, we analyzed the interaction of the Hbp signal peptide with SRP using an *in vitro* translation and crosslinking assay. Of note, crosslinking of the SRP component Ffh (for fifty four homologue) to short nascent polypeptides is a reliable indication for functional interactions with the SRP [15]. Radiolabeled nascent chains of Hbp and Hbp[ΔExt] were generated from truncated mRNA in a cell- and membrane free *E. coli* lysate. Nascent chain lengths of 116 (Hbp) and 93 (Hbp[ΔExt]) residues were chosen to provide optimal exposure of the signal peptides at the ribosome, and to warrant identical positioning of the respective signal peptides relative to

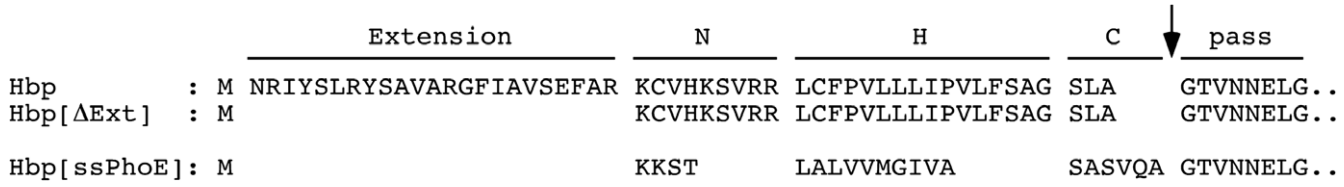

Fig. 1. Hbp derivatives used in this study. Primary sequence of the N-terminus of Hbp, Hbp[ΔExt] and Hbp[ssPhoE] including the signal peptide and the first eight residues of the Hbp passenger (*pass*). The SPaseI cleavage site is indicated with an arrow.