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A signal peptide with a proline next to the cleavage site inhibits leader peptidase when present in a *sec*-independent protein

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Proline residues are rarely found in the three most C-terminal positions of bacterial signal peptides, and have never been found in position +1 immediately following the cleavage site. It was recently shown that a Pro^{+1} mutation in the *E. coli* maltose binding protein precursor not only prevents cleavage of the signal peptide but also inhibits the leader peptidase enzyme, resulting in cessation of cell growth (Barkocy-Gallagher, G.A. and Bassford, P.J. (1992) J. Biol. Chem. (in press)). Since maltose binding protein is dependent on the *sec* machinery for translocation across the inner membrane, it was not clear if this 'Pro^{+1}' effect was restricted to *sec*-dependent proteins, or whether it applies also to proteins that do not require the *sec* functions for translocation. We now present data suggesting that the striking phenotypic effects of Pro^{+1} mutations can be elicited also by *sec*-independent proteins.

Leader peptidase; Protein secretion; Signal peptide; *sec* pathway

1. INTRODUCTION

Amino-terminal signal peptides serve to target proteins for secretion in both prokaryotic and eukaryotic cells. A number of statistical as well as experimental studies [2,3] point to a tripartite design as being central for signal peptide function: an N-terminal, positively charged region (n-region), a middle, hydrophobic region (h-region), and a C-terminal region containing the signal peptidase recognition site (c-region). In *Escherichia coli*, signal peptides normally trigger the *sec*-dependent translocation machinery (minimally defined by the SecA, SecD, SecE, SecF and SecY proteins [4]), although a small number of proteins are known not to require a functional *sec* pathway for translocation [5–8].

In *E. coli*, most signal peptides are cleaved from the nascent chain by the inner membrane enzyme leader peptidase (Lep; also called signal peptidase I) [9]. In part, the substrate specificity of Lep is described by the so-called (−3,−1)-rule [10–14]: only small, neutral amino acids are allowed in the −3 and −1 positions relative to the cleavage site. Beyond the (−3,−1)-rule, however, other amino acid preferences have also been noted; in particular, proline is rarely found in positions −3 to −1, and has never been observed in position +1 (i.e. as the first residue of the mature chain) in bacterial signal peptides ([12], and our unpublished data). Recent mutational analysis of the *E. coli* maltose binding protein (MBP) signal peptide has revealed not only that the introduction of a Pro in position +1 completely blocks processing in vivo, but further that the Pro^{+1} signal peptide seems to act as a competitive inhibitor of the leader peptidase enzyme [1].

As translocation of MBP to the periplasm is dependent on the *sec* machinery [4], we wanted to test whether the same effects on Lep activity would be elicited when a Pro^{+1} mutation is introduced in an unrelated protein that is *sec*-independent. We have previously found that a 'consensus cleavage cassette' embodying the known statistical amino acid preferences of *E. coli* signal peptide c-regions (positions −6 to +2) is efficiently cleaved at the intended site by Lep when introduced after normally uncleaved N-terminal hydrophobic transmembrane anchor segments [15]. Now we show, first, that this cleavage cassette is processed also when present in a protein that does not depend on the *sec* machinery for translocation, and, second, that the introduction of a Pro^{+1} mutation in this protein results in the same phenotype as observed for the MBP Pro^{+1} signal peptide mutant (prevention of cleavage, partial inhibition of leader peptidase activity and impaired cell growth). These observations make it highly likely that the detrimental effect on leader peptidase activity and cell growth of Pro^{+1} signal peptide mutations is a general phenomenon, and, more importantly, that there is only one pool of the leader peptidase enzyme that acts identically on proteins that are delivered through the *sec*-dependent and *sec*-independent pathways.

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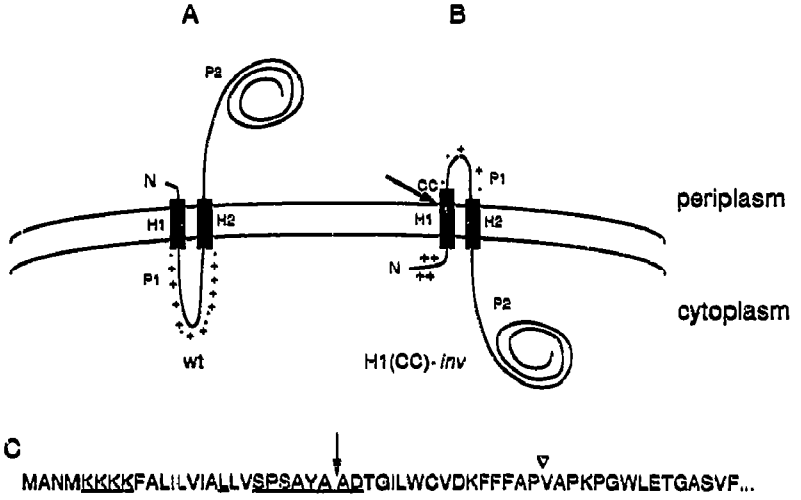

Fig. 1. (A) Wild-type Lep. (B) Lep H1(CC)-*inv*. (C) Sequence of the N-terminal region of H1(CC)-*inv* up to the beginning of H2. In H1(CC)-*inv*, 4 lysines have been added between residues 4 and 5 to the N-terminus of the H1 transmembrane region (residues 4–22), Thr^{13} in H1 has been changed to Leu, a 'cleavage cassette' (CC, black) has been added to H1 between residues 15 and 16, and residues 30–52 in P1 have been deleted, converting H1 into a cleavable signal sequence [8,15]. In (C), residues not present in wild-type Lep are underlined, the Lep cleavage-site in the cleavage cassette is indicated by an arrow, and the position of the Δ30–52 deletion is shown (∇).

2. MATERIALS AND METHODS

2.1. *Enzymes and chemicals*

Unless otherwise stated, all enzymes were from Promega. [^{35}S]Methionine was from Amersham. Ribonucleotides, deoxyribonucleotides, and dideoxyribonucleotides were from Pharmacia. Oligonucleotides were produced using an Applied Biosystems synthesizer 380B followed by NAP-5 (Pharmacia) purification. Pansorbin was from CalBiochem.

2.2. *Strains and plasmids*

Leader peptidase mutants were expressed from the pING1 plasmid [16] in *E. coli* strain MC1061 [17].

2.3. *DNA techniques*

Site-specific mutagenesis was performed according to the method of Kunkel [18], as modified by Geisselsoder et al. [19]. All mutants were confirmed by DNA sequencing of single-stranded M13 DNA using T7 DNA polymerase (Pharmacia). Cloning into the pING1 plasmid was performed as described in [20].

2.4. *Radiolabelling experiments*

E. coli strains transformed with the pING1 vector carrying mutant leader peptidase (*lep*) genes under control of the *ara* promoter were grown at 37°C in M9 minimal medium supplemented with 100 μg/ml ampicillin, 0.5% fructose, and all amino acids (50 μg/ml each) except methionine. Overnight cultures were diluted 1:35 in 1 ml fresh medium, shaken for 3.5 h at 37°C, induced with arabinose (0.2%) for 5 min, and labelled with [^{35}S]methionine (30 μCi/ml). After the indicated labelling times, non-radioactive methionine was added (500 μg/ml) and incubation was stopped by acid-precipitation (ice-cold trichloroacetic acid, 10% final conc.). Cells were resuspended in 10 mM Tris/2% SDS, immunoprecipitated with antisera to Lep and OmpA [21], adsorbed onto Pansorbin, washed, and analyzed by SDS-PAGE and fluorography.

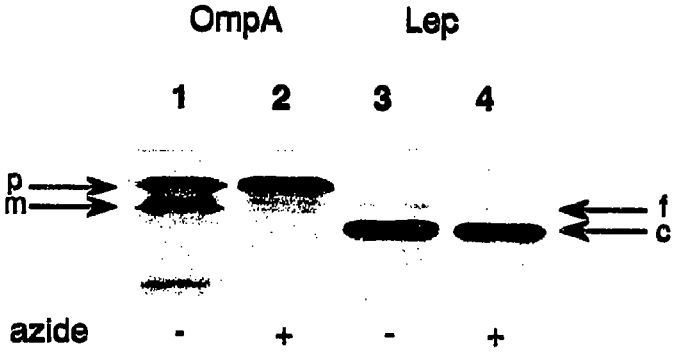

Fig. 2. Signal peptide cleavage of H1(CC)-*inv* is SecA-independent. Production of mutant proteins was induced with arabinose for 4 min, SecA function was blocked by incubation with 1 mM sodium azide for an additional 1 min (lanes 2, 4), cells were labelled with [^{35}S]methionine for 15 s, and immunoprecipitated with antisera to OmpA and Lep. Full-length (f) and cleaved (c) forms of Lep as well as the precursor (p) and mature (m) forms of the *sec*-dependent control OmpA are indicated.

3. RESULTS

3.1. *A consensus signal peptide cleavage cassette functions in a sec-independent context*

We have previously shown [15] that a 'consensus cleavage cassette' with the sequence Ser-Pro-Ser-Ala-Tyr-Ala^{-1}↓Ala^{+1}-Asp is efficiently cleaved by Lep at the intended site (arrow) when placed after either of two unrelated, normally uncleaved internal signal sequences in constructs derived from Lep itself [15]. However, translocation is *sec*-dependent for both of these constructs (data not shown), and we thus sought to introduce the cleavage cassette in a *sec*-independent context.

Studies on the role of positively charged residues during membrane protein insertion have demonstrated that the orientation of proteins from the inner membrane of *E. coli* can be manipulated by repositioning of such residues relative to the apolar transmembrane segments

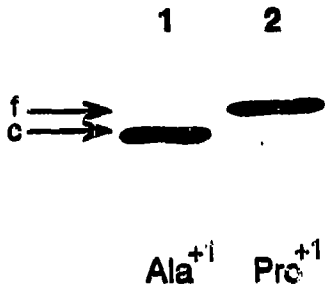

Fig. 3. Pro^{+1} inhibits processing of the consensus cleavage cassette in H1(CC)-*inv*. Cells were induced for synthesis of mutant protein with arabinose for 5 min, then labelled with [^{35}S]methionine for 30 s and immunoprecipitated with Lep antiserum. (Lane 1) H1(CC)-*inv* Ala^{+1}. (Lane 2) H1(CC)-*inv* Pro^{+1}. Full-length (f) and cleaved (c) forms are indicated.

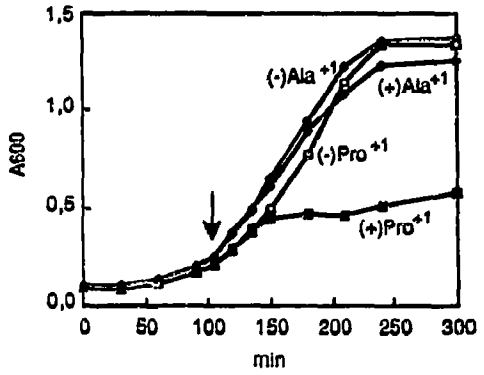

Fig. 4. Pro^{+1} inhibits cell growth when present in a *sec*-independent construct. Cells were grown in amino acid- and fructose-supplemented M9 minimal medium and induced for synthesis of mutant protein with arabinose at the indicated time (105 min). Growth was followed by measuring the optical absorbance at 600 nm. Growth curves for cells induced (+, filled symbols) and not induced (–, open symbols) for synthesis of H1(CC)-*inv* Ala^{+1} (◇ ◆) and Pro^{+1} (□ ■) are shown.

[22–24]. In particular, the orientation of Lep can be 'inverted' by adding lysines to the N-terminal end of the H1 transmembrane segment while simultaneously deleting a highly charged segment from the P1 domain [8,25,26] (Fig. 1). In contrast to wild-type Lep, the resulting molecule, Lep'-*inv*, inserts across the membrane in a *sec*-independent fashion [8].

The cleavage cassette was introduced towards the C-terminal end of the H1 segment in a construct closely related to Lep'-*inv*, only lacking a 9-residue peptide originally inserted into the P1 domain to increase its sensitivity to digestion by externally added trypsin [8]. This mutant, called H1(CC)-*inv*, thus differs from wild-type Lep in three regions: it has four extra lysines inserted between codons 4 and 5, it has a cleavage cassette Ser-Pro-Ser-Ala-Tyr-Ala^{-1}↓Ala^{+1}-Asp inserted between codons 15 and 16 (and codon 13 has been changed from Thr to Leu; this change significantly increases the fraction of cleaved product, see below), and residues 30–52 have been deleted from the P1 domain leaving 25 residues between the consensus cleavage-site and H2 (Fig. 1).

H1(CC)-*inv* was cloned into plasmid pING1, where it is placed under control of the inducible *ara* promoter [16]. When overproduced in *E. coli*, most of the protein is present as a cleaved form with a mol. wt. consistent with cleavage at the intended site by the chromosomally encoded wild-type Lep (Fig. 2, lane 3) (cf. Fig. 3; also note that the level of wild-type Lep is too low to be seen on our gels). Furthermore, inhibition of SecA function by incubation in 1 mM sodium azide [27], while efficiently blocking cleavage of the precursor form of the *sec*-dependent [28] outer membrane protein, OmpA, has no effect on the cleavage of H1(CC)-*inv* (Fig. 2, lanes 2, 4). Similar results were obtained when H1(CC)-*inv* was expressed at the non-permissive temperature in strain CU164 which is SecY^{cs} [29] (data not shown). We conclude that H1(CC)-*inv* is cleaved by Lep and inserts across the inner membrane in a *sec*-independent fashion.

3.2. *Pro^{+1} blocks processing of the consensus cleavage cassette*

Since a proline residue in position +1 (i.e. immediately C-terminal to the signal peptide cleavage site) has been reported to block cleavage of the signal peptide in MBP, ribose binding protein, OmpA and β-lactamase [1,30,31], all of which are *sec*-dependent for translocation, we tested its effects in the *sec*-independent construct H1(CC)-*inv*. As shown in Fig. 3, processing is completely abolished when Ala^{+1} in the cleavage cassette is changed to Pro, as expected if the cleavage cassette is indeed cleaved by Lep at the same site where it was previously shown to be cleaved in other, *sec*-dependent Lep constructs [15].

3.3. *Pro^{+1} inhibits cell growth and leader peptidase activity when placed in a sec-independent context*

As has been observed for MBP [1], induction of the H1(CC)-*inv* Pro^{+1} mutant (but not the Ala^{+1} parent construct) leads to cessation of cell growth within 1 h (Fig. 4). Further, again corresponding to the results reported for MBP, the kinetics of cleavage of the *sec*-dependent OmpA precursor is significantly affected already 5 min after induction of the Pro^{+1} mutant (Fig. 5), but is largely unaffected by induction of the corresponding Ala^{+1} construct. Thus, a Pro^{+1} mutant signal peptide appears to have very similar phenotypic effects irrespective of context and irrespective of whether it is inserted into the membrane via the *sec*-dependent or *sec*-independent pathways.

4. DISCUSSION

Earlier studies on an MBP mutant with a proline in position +1 next to the signal peptide demonstrated that the mutant protein acts as a competitive inhibitor of the

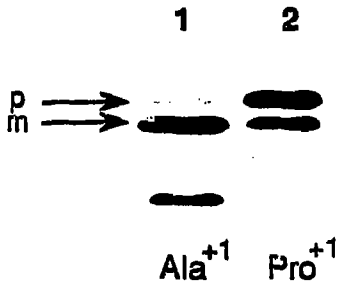

Fig. 5. Induction of the H1(CC)-*inv* Pro^{+1} mutant inhibits leader peptidase activity. Cells were induced for synthesis of mutant protein with arabinose for 5 min, then labelled with [^{35}S]methionine for 30 s and immunoprecipitated with OmpA antiserum. (Lane 1) H1(CC)-*inv* Ala^{+1}. (Lane 2) H1(CC)-*inv* Pro^{+1}. OmpA precursor (p) and mature (m) forms are indicated.

leader peptidase enzyme by a number of criteria [1]: (i) the processing of other precursors to exported proteins normally cleaved by Lep was inhibited; (ii) processing of precursors cleaved by signal peptidase II (Lpp) was not affected; (iii) the phenotypic effects were only observed with an otherwise functional signal peptide that supported translocation of the precursor across the inner membrane; (iv) secondary mutations in position −1 that by themselves block processing by Lep restored normal growth; and (v) overproduction of wild-type Lep also corrected the Pro^{+1} defects.

Our results show that placing a proline in position +1 of an artificial consensus cleavage cassette in a *sec*-independent protein leads to similar phenotypic effects (cessation of cell growth, inhibition of processing of the OmpA precursor), suggesting that also in this case the Pro^{+1} signal peptide acts as a competitive inhibitor of Lep rather than via some indirect effects on secretion. This indicates that there are no separate pools of Lep that are specific for proteins routed, respectively, through the *sec*-dependent and *sec*-independent pathways, since the *sec*-independent H1(CC)-*inv* Pro^{+1} mutant would not be expected to affect processing of the *sec*-dependent OmpA protein if this was the case. A priori, one could imagine that the SecA/Y/E translocation complex would be physically associated with a fraction of the Lep molecules that would only bind to and cleave precursor proteins emerging through the *sec* pathway; our results rather suggest that Lep is not complexed to the *sec* machinery and cannot distinguish between *sec*-dependent and *sec*-independent precursor proteins.

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