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Requirement for Signal Peptide Cleavage of *Escherichia coli* Prolipoprotein

Abstract. *Oligonucleotide-directed site-specific mutagenesis was applied to alter the cleavage site in the signal peptide of the major outer membrane lipoprotein of* Escherichia coli. *Replacing the glycine residue at the cleavage site with an alanine residue did not affect the processing of the signal peptide. However, when the same cleavage site was constructed by the deletion of the glycine residue, the signal peptide was no longer cleaved. These results indicate that stringent structural integrity at the cleavage site in the lipoprotein signal sequence is required for correct processing of prolipoprotein.*

Secretory proteins are initially synthesized in the cytoplasm as higher molecular weight precursors that contain an extra peptide extension (signal sequence) at their amino terminals (*1*). In contrast to eukaryotic signal peptides, prokaryotic signal peptides show several major structural homologies including (i) one to three basic amino acid residues at the amino terminal, (ii) a sequence of 10 to 15 hydrophobic amino acids directly following the positively charged amino terminus, (iii) a serine or threonine residue (or both) following the hydrophobic core and located close to the carboxyl terminal, (iv) an alanine or glycine residue at the signal peptide cleavage site, and (v) in most signal peptides, a proline or glycine residue within the hydrophobic domain (*1*, *2*). These common features were incorporated in a model (loop model) originally proposed to explain the functions of the signal peptide (*1*, *3*). We have investigated the structural requirements at the signal peptide cleavage site of the precursor of the major outer membrane lipoprotein, the prolipoprotein, of *Escherichia coli*.

The prolipoprotein signal peptide consists of 20 amino acid residues with the sequence

1 5
Met-Lys-Ala-Thr-Lys-Leu-Val-
10 15
Leu-Gly-Ala-Val-Ile-Leu-Gly-Ser-
20 ↓ 21
Thr-Leu-Leu-Ala-Gly-Cys-

where an arrow indicates the position of the cleavage site (*3*, *4*). The positively charged amino terminal region plays an important role in efficient protein secretion across the cytoplasmic membrane (*2*, *5*). We demonstrated earlier that the cleavage of the signal peptide was completely blocked if a glycine residue replaced the cysteine residue (*6*), indicating that the cysteine residue is essential for the signal peptide to be cleaved by the prolipoprotein signal peptidase (*7*). Because all prolipoproteins so far characterized have a glycine residue at the cleavage site (*2*), we examined whether the prolipoprotein signal peptidase can catalyze the cleavage of the linkage between the alanine and cysteine residues instead of the linkage between glycine and cysteine. This was achieved both by replacing the glycine residue at position 20 with an alanine residue and by deleting the same glycine residue. In order to obtain such mutations, we guided site-specific mutagenesis using as mutagens the following synthetic oligodeoxyribonucleotides (*4*).

Oligonucleotide 1:
5′ CTG GCA GCT TGC TCC 3′
Leu Ala Ala Cys Ser

Oligonucleotide 2:
5′ TG CTG GCA TGC TCC AGC 3′
Leu Leu Ala Cys Ser Ser

Oligonucleotide 1 is designed to cause a single base substitution in the codon for the glycine residue at the cleavage site from GGT to GCT, a codon for alanine. Oligonucleotide 2 is designed to cause a deletion of three bases GGT, the codon for the same glycine.

Guided site-specific mutagenesis was carried out as described (*2*) with the use of plasmid pMI001, a clone of the structure gene of the prolipoprotein (*5*). Five of 392 transformants after treatment with oligonucleotide 1, and 2 of 784 transformants after treatment with oligonucleotide 2, had the desired mutations. Plasmids from one of the positive colonies from each mutagenesis were further characterized by DNA sequence analysis (Fig. 1). The sequences of the mutant DNA's were identical to those predicted from the mutagenesis design. Since pMI001 had no promoter, a 0.56–kilobase (kb) Xba I–Eco RI fragment carrying the mutant *lpp* gene was isolated from each mutant plasmid and inserted into an expression cloning vector, pIN-II (*8*), which was digested with both Xba I and Eco RI. The resultant plasmids, pC_1 [pIN-II-*lpp* ($Gly^{20} \rightarrow Ala$)] and pC_3 [pIN-II-*lpp* (ΔGly^{20})], now had the mutant *lpp* gene under the control of the *lpp* promoter and the *lac*UV5 promoter-operator (*6*, *8*). Thus, the expression of the mutant *lpp* genes could be induced by a *lac* inducer such as isopropyl-β-D-thio-

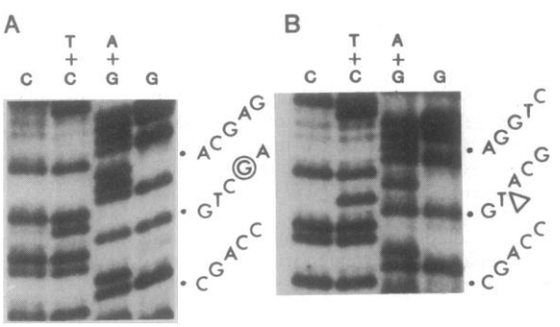

Fig. 1. DNA sequence gels corresponding to the region of the cleavage site of the prolipoprotein signal peptide of pC_1 [pIN-II-*lpp* ($Gly^{20} \rightarrow Ala$)] and pC_3 [pIN-II-*lpp* (ΔGly^{20})]. The plasmid DNA was digested with Xba I at the unique Xba I site in the ribosome-binding site of the *lpp* gene and labeled with deoxynucleotide [^{32}P]triphosphate (*5*). The labeled DNA was cleaved with Hinf I and the approximately 120-base pair Xba I–Hinf I fragment was purified and used for sequencing (*14*). The cleavage products were applied to a 10 percent polyacrylamide gel and separated by electrophoresis. Only the region around the signal peptide cleavage site is shown. The sequences at the side of the gel are complementary to the sense sequence. The circled base indicates the base that is different from the wild-type *lpp* gene. A triangle indicates a deletion of a sequence of the three bases CCA. (A) pC_1 DNA and (B) pC_3 DNA.

galactoside (IPTG). *Escherichia coli* JA221 *lpp*$^-$/F'*lacI*^{q} (*9*) carrying pC_1 was not sensitive to IPTG, whereas *E. coli* JA221 *lpp*$^-$/F'*lacI*^{q} carrying pC_3 was sensitive to the *lac* inducer and could not form colonies on a plate containing IPTG. These results indicate that replacing the glycine residue at position 20 with an alanine residue did not cause any deleterious effects to the cell, whereas the deletion of the same glycine residue had a lethal effect on the cell.

For further characterization, exponentially growing cells were labeled for 30 seconds with [^{35}S]methionine following a 20-minute induction with IPTG. After the cells were immunoprecipitated with antiserum to lipoprotein, the soluble and the membrane fractions were analyzed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis used with a sodium phosphate system (*5*, *6*). The product from pC_1 appeared to be the fully modified mature lipoprotein assembled in the membrane fraction (lane 3 in Fig. 2). In contrast, the product from pC_3 was identified as the unmodified mutant prolipoprotein by its mobility in SDS-polyacrylamide gels used with the sodium phosphate and tris-HCl buffer systems (data not shown). Most of this product was found in the membrane fraction (lane 4 in Fig. 2); the mature lipoprotein was not detected in either the soluble (lane 2 in Fig. 2) or the membrane fraction. Even if cells that had been labeled for only 2 minutes were further incubated for 30 or 60 minutes with nonlabeled methionine, the mature lipoprotein was not detected; under these circumstances the unmodified mutant prolipoprotein was unstable and quickly degraded (data not shown). The fact that the pC_3 mutant prolipoprotein was not modified with a glyceride group or a glycerol group was shown by its ability to form a dimer in the absence of β-mercaptoethanol (data not shown). The dimer can be formed only if the cysteine residue at position 21 is not modified at its sulfhydryl group. In contrast, the processing of the pC_1 prolipoprotein was inhibited by globomycin (50 μg/ml) as is the wild-type prolipoprotein (*10*), and the accumulated pC_1 prolipoprotein was normally labeled with [^{3}H]palmitic acid (data not shown). These results indicate that the pC_1 prolipoprotein, fully modified with a glyceride group, is specifically processed by the prolipoprotein signal peptidase (*7*).

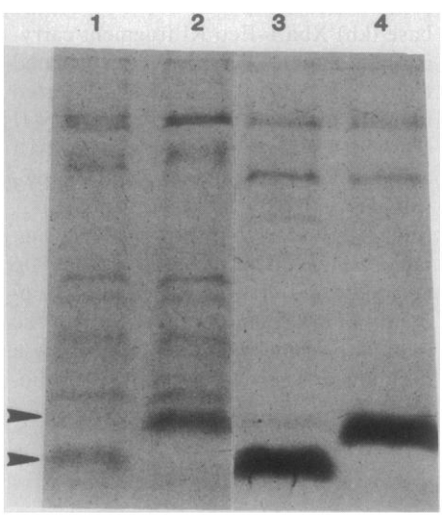

Fig. 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of immunoprecipitates with antiserum to lipoprotein used with the sodium phosphate system (*6*). *Escherichia coli* JA221 *lpp*$^-$/F'*lacI*^{q} carrying pC_1 or pC_3 were pulse-labeled with [^{35}S]methionine (*5*). The soluble and the membrane fractions were separated and treated with antiserum to lipoprotein (*5*). (Lane 1) The soluble fraction of cells carrying pC_1; (lane 2) the soluble fraction of cells carrying pC_3; (lane 3) the membrane fraction of cells carrying pC_1; and (lane 4) the membrane fraction of cells carrying pC_3. The top arrow indicates the position of the unmodified prolipoprotein and the bottom arrow the position of the mature lipoprotein (*6*).

The pC_1 lipoprotein was found almost exclusively in the outer membrane fraction as in the case of the wild-type lipoprotein (data not shown). The pC_3 prolipoprotein was also almost exclusively localized in the outer membrane, as separated by Sarkosyl differential solubilization (Fig. 3). The same result was obtained when the cytoplasmic and outer membranes were separated by sucrose-density gradient centrifugation (data not shown).

Our results indicate that the prolipoprotein signal peptidase (*7*) can cleave the peptide bond not only between glycine and glyceride-modified cysteine residues but also between alanine and the glyceride-cysteine residues. In addition, the glyceride modification reactions at the cysteine residue were not affected by this substitution mutation. However, when the glycine residue at the cleavage site was deleted, the mutant prolipoprotein was incapable of being modified with a glyceride group or processed by the prolipoprotein signal peptidase. The glycine deletion resulted in an alanine residue at the cleavage site, as in the case of the pC_1 mutation. The effect of the glycine deletion is not due simply to the shortening of the hydrophobic region of the signal peptide by one amino acid residue, since the mutant prolipoprotein with the deletion of a glycine residue at position 9 or 14 could still be modified and processed to form the mature lipoprotein assembled in the outer membrane (*11*). This indicates that the structure Ser-Thr-Leu-Leu-Ala-Gly (residues 15 to 20) may play an important role in forming a specific conformation required for the prolipoprotein modification and the cleavage of the signal peptide.

The effect of the pC_3 mutation on prolipoprotein processing appears to be similar to that of the mutation resulting from substituting an aspartic acid residue for the glycine residue at position 14 (*12*). However, the pC_3 mutant prolipoprotein was found almost exclusively in the outer membrane fraction, whereas the position 14 mutant prolipoprotein was found not only in the outer membrane but also in the cytoplasmic membrane and in the cytoplasm (*12*). The results with the pC_3 mutation therefore clearly indicate that the signal peptide of a secretory protein has two distinct functions: (i) the translocation of the secretory protein from the inside to the outside of the cytoplasmic membrane and (ii) the formation of a

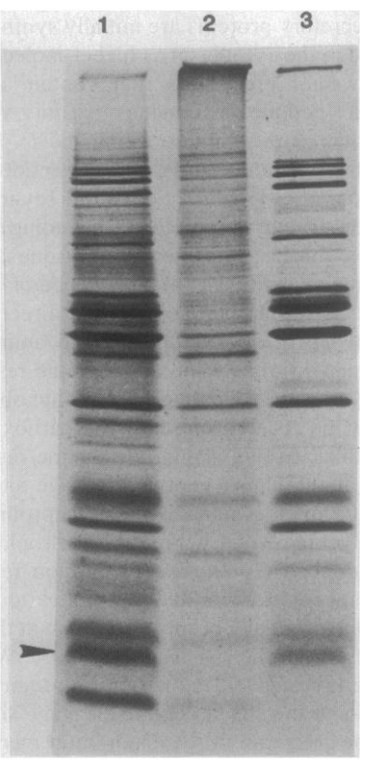

Fig. 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the cytoplasmic and outer membrane fractions of *E. coli* JA221 *lpp*$^-$/F'*lacI*^{q} harboring pC_3. The cells were labeled with [^{35}S]methionine for 2 minutes after a 20-minute induction with IPTG as in Fig. 2. The membrane fraction was prepared and treated with sodium sarcosynate to separate the soluble cytoplasmic and insoluble outer membrane fractions (*15*). These fractions were subjected to SDS-polyacrylamide gel electrophoresis used with a tris-HCl system. (Lane 1) The total membrane fraction; (lane 2) the cytoplasmic; and (lane 3) the outer membrane fractions. An arrow indicates the position of the unmodified prolipoprotein.

specific conformation to serve as a substrate for a signal peptidase. Since the second function is not required for the first, a secretory protein can be translocated across the cytoplasmic membrane without the cleavage of the signal peptide. Therefore, inhibition of signal peptide cleavage results in the accumulation of a secretory precursor protein, which is attached to the cytoplasmic membrane through the signal peptide. However, if the precursor protein has a strong affinity for the outer membrane, as in the case of the pC_3 mutant prolipoprotein, it may be pulled out of the cytoplasmic membrane and assembled in the outer membrane with the signal peptide uncleaved. Similar results have been observed in a mutant in which the cysteine residue was replaced with a glycine residue (*6*) and in a case in which prolipoprotein accumulated in the presence of globomycin (*13*).

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Induction of Neuronal Branching in *Caenorhabditis elegans*

Abstract. *The two postembryonic touch receptor neurons in the nematode* Caenorhabditis elegans *arise from essentially identical cell lineages and have the same ultrastructural features. The cells are found in different positions in the animal, however, and differ in neuronal branching, connectivity, and function. These structural and functional differences are not seen when cells are placed in similar positions by mutation or laser-induced damage. Thus, some, but probably not all, of the differentiated properties of these cells are a consequence of their cellular environment.*

Little is known about how much of neuronal structure is the product of an intrinsic developmental program within a given cell and how much is the result of cellular interactions during maturation (*1*). In this report we examine the consequences of ectopic placement of one set of neurons, the touch receptor cells, in the nematode *Caenorhabditis elegans* on their subsequent development. The touch cells were repositioned by two methods. In the first, advantage was taken of a mutation that affects the migration of one of the touch cell precursors, and in the second the migration of the precursor was blocked by a barrier of laser-produced debris. We found that some, but not all, of the structural features of the cells depended on cell position. Specifically, neuronal branching appeared to be induced only when the cells were in the correct position within the animal, but the length of the neuronal process and ultrastructural features of the process were not affected by cell position.

The development and structure of the *C. elegans* nervous system have been extensively studied over the past few years (*2–10*). As a result of these studies the cell lineage origins (*9*) and structure (*10*) of all of the 302 neurons of the adult hermaphrodite have been described. Almost all of the nerve cells are structurally simple; cells are either monopolar or bipolar and lack extensive arborizations. Most neuronal processes are unbranched, although a few show one or two branches.

The touch receptor cells (the microtubule cells) have a simple branching pattern (*11, 12*) (Fig. 1). Each cell has a single long receptor process that projects anteriorly from the cell body. This process is filled with a unique class of microtubules that give the cells their name. In addition, the receptor process has associated extracellular material (the mantle) that is also characteristic of the cells. These features are probably important for sensory transduction along the entire length of the receptor process (*12, 13*). Near the end of the receptor process in most touch cells is a branch (the synaptic branch) on which a number of synapses are made. There are six microtubule cells in *C. elegans*, but in this paper we will discuss primarily the two ventral cells—the anterior ventral microtubule (AVM) cell and the posterior ventral microtubule (PVM) cell.

The AVM and PVM arise postembryonically from essentially identical cell lineages (*7*). The AVM is produced on the right side of the animal from divisions of cell Q2, and PVM is produced

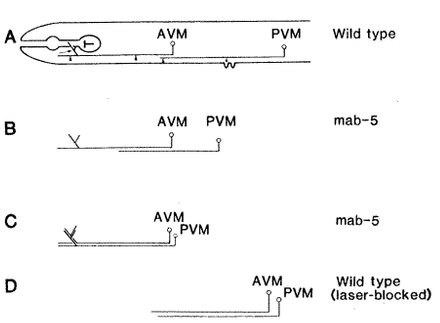

Fig. 1. Positions of AVM and PVM under various conditions. The position of the receptor processes (triangles) and the AVM synaptic branch (arrow) are indicated. Except in wild type (A), the complete structure of the synaptic branches is not known; electron micrographs of serial sections (*2*) of the region of the wild-type synaptic branch have been examined only to the extent of determining whether branching had occurred. The lengths of the receptor processes were estimated from the extent of microtubule-containing processes in electron microscopic sections (a short segment at the end of the process lacks microtubules, but is ignored here). (A) Wild type (*C. elegans* var. Bristol; strain N2). (B) Mutant *mab-5* animals in which PVM is located in the middle of the animals. The *mab-5* stock used in all these experiments (CB3531) contained, in addition to the *mab-5(e1239)III* mutation, a *him-5(e1490)V* mutation (*22*). (The *mab-5* mutation affects male tail structure, and the *him* mutation increases the frequency of males so that this effect is more apparent.) (C) Mutant *mab-5* animals in which PVM is located in the anterior ventral cord. (D) Wild-type animals in which the migration of Q2 (the precursor to AVM) has been blocked by debris produced by laser damage to the hypodermis (*21*). It was difficult to block the migration of Q2 without damaging the cells. The best results were obtained when cell V4 and the adjacent hypodermal nucleus (*7*) were killed in newly hatched larvae.