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Site-Specific Proteolysis of the *Escherichia coli* SecA Protein In Vivo

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A seven-amino-acid cleavage site specific for tobacco etch virus (TEV) protease was introduced into SecA at two separate positions after amino acids 195 and 252. Chromosomal wild-type *secA* was replaced by these *secA* constructs. Simultaneous expression of TEV protease led to cleavage of both SecA derivatives. In the functional SecA dimer, proteolysis directly indicated surface exposure of the TEV protease cleavage sites. Cleavage of SecA near residue 195 generated an unstable proteolysis product and a secretion defect, suggesting that this approach could be used to inactivate essential proteins in vivo.

SecA is an essential *Escherichia coli* protein involved in secretion (for a review, see reference 15). It binds precursor proteins and precursor-SecB complexes that are subsequently delivered to the integral membrane proteins SecY and SecE. While interacting with SecYE, SecA is membrane associated and is accessible from the periplasm (5, 9). SecA is active as a homodimer (4), binding and hydrolyzing ATP, a process which is required for translocation. The relevant ATP-binding domains have been identified and characterized in detail (14). The high-affinity ATP binding site is located at residues 102 to 109 (A0 motif) and 198 to 210 (B0 motif). A second, low-affinity site is composed of residues 503 to 511 and 631 to 653. Both ATP-binding domains are essential for SecA function. Truncated amino (N)- and carboxy (C)-terminal SecA fragments have the ability to bind ATP when they are combined but not when they are present individually. Although ATP binds, complementation of translocation does not occur in the reassembled N- and C-terminal fragments (12).

From cross-linking experiments, a preprotein binding site between amino acid residues 267 and 340 (10) was postulated. Not much else is known about the structure of SecA. Although three-dimensional crystals can be raised, they do not diffract (19). In a linker insertion mutagenesis experiment originally set up to identify regions which are essential for function, two insertions after codons 195 and 252 were generated which do not inactivate SecA (8). Linker insertions which do not disrupt target protein function are presumed to be located in surface-exposed loops. However, linker insertions provide only indirect evidence for surface accessibility. We exchanged the existing linker insertions in SecA with those containing a recognition sequence for a site-specific protease. If coexpression of the protease leads to a cleaved target protein, direct evidence for surface exposure of these linkers could thus be obtained. It was also interesting to test whether SecA function is disrupted by site-specific proteolysis.

We used tobacco etch virus (TEV) protease (for a review, see reference 3), which recognizes a seven-amino-acid consensus sequence, Glu-X-X-Tyr-X-Gln-Ser/Gly, where X can be almost any amino acyl residue (2). Cleavage occurs between the conserved Gln and Ser/Gly residues (1). Polypeptides which are not a natural substrate of TEV protease are proteolyzed if they carry the appropriate cleavage site. It has also been shown that TEV protease can be functionally expressed in yeast cells without interfering with viability (18).

Insertion of the TEV protease cleavage site into SecA. We used Glu-Asn-Leu-Tyr-Phe-Gln-Ser as a TEV protease cleavage site. The site was inserted into *secA* clones pGJ4 and pGJ11 (8) containing *Apa*I linker insertions after codons 195 and 252, respectively (Fig. 1). These *secA195* and *secA252* derivatives were constructed by cloning of the annealed primers GAAAAC CTGTACTTCCAGTCAGATCTGGCC and AGATCTGACT GGAAGTACAGGTTTTCGGCC into the *Apa*I restriction site of pGJ4 and by cloning of the annealed primers CTGAA AACCTGTACTTCCAGTCAGATCTGGGCC and CAGATC TGACTGGAAGTACAGGTTTTCAGGGCC into the *Apa*I restriction site of pGJ11.

These constructs were transformed into strain MM52 [MC4100 *secA*(Ts) (16)]. MM52 does not grow at 42°C because of its conditional SecA defect. MM52 expressing the plasmid-encoded SecA195 and SecA252 derivatives grew well at 42°C. Thus, SecA function was not abolished by the insertions containing TEV protease cleavage sites. Subsequently, chromosomal wild-type *secA* was replaced by the *secA195* and *secA252* alleles by linear transformation with a *recD*::Tn*10* derivative of MM52. Recombinants were selected for growth at 42°C, and the replacement of chromosomal *secA*(Ts) by *secA195* and *secA252* was verified by detection of a larger SecA protein on Western blots (immunoblots). In addition, PCRs were carried out with single colonies and one primer, TGTCGGTAT CAACCTGC (starting at nucleotide 459 of *secA*), as well as the individual primers GTACAGAGACTCCCCTT (starting at nucleotide 897 of *secA*) and GAAAACCTGTACTTC CAGTCAGATCTGGCC (complementary to the inserted sequence encoding the protease cleavage site) (data not shown). Subsequently, *secA195* and *secA252* were transduced into strain MM52 by P1 transduction, with selection for growth at 42°C. As in the case of the plasmid-encoded *secA195* and *secA252* alleles, no growth defects were detected at either 28, 37, or 42°C. In the absence of TEV protease, protein encoded by the *secA195* or *secA252* alleles was stably expressed, as was shown in a pulse-chase experiment (data not shown).

Coexpression of *secA195* and TEV protease. A *secA195* derivative of strain MC4100 was transformed with pGEX-2T expressing a glutathione *S*-transferase (GST)-TEV protease hybrid protein (17). SecA195 was cleaved by TEV protease in vivo. A proteolytic fragment of 85 kDa, corresponding to the SecA C terminus, was detected on Western blots after cells

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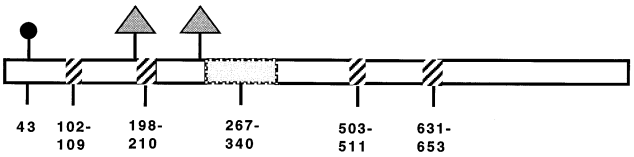

FIG. 1. Schematic map of SecA. The schematic map of SecA shows the positions of the introduced TEV protease cleavage sites (shaded triangles). The A0 and B0 motifs of the high-affinity ATPase domain are located between amino acids 102 and 109 and 198 and 210, respectively. The postulated precursor binding site is located between amino acids 267 and 340. The A0 and B0 motifs of the low-affinity ATPase domain are located between amino acids 503 and 511 and 631 and 653, respectively. The temperature-sensitive phenotype of strain MM52 is caused by an L43P mutation (solid circle).

were grown to the stationary phase. However, we could not detect the other cleavage product with a size of 21 kDa, which may be explained by either a degradation of this fragment by cellular proteases or the inability of the polyclonal antibody to recognize the N terminus of SecA (data not shown). Subsequently, we tested the stability of the proteolytic fragments in a pulse-chase experiment.

Protein was labeled in cultures of strain MC4100 expressing either wild-type SecA or SecA195 from the chromosome and TEV protease from pGEX-2T growing exponentially (optical density at 600 nm, 0.4) in minimal medium 9 (13) (0.2% glucose, 1 μg of thiamine per ml, 50 μg of ampicillin per ml, 10 μM IPTG [isopropyl-β-D-thiogalactopyranoside]) to induce expression of TEV protease, supplemented with each common amino acid except cysteine and methionine. One milliliter of cells was exposed to [^{35}S]methionine (>1,000 Ci/mmol) at 10 μCi/ml for 45 s and subsequently cooled on ice for immunoprecipitation, or the labeling period was followed by a chase with excess cold methionine (final concentration, 50 mM) for the time indicated. Immunoprecipitation and gel electrophoresis were done as described by Ito et al. (7) and Laemmli (11). To precipitate SecA, polyclonal antibodies against SecA were used.

We observed that the proteolytic fragment with a size of 85 kDa was unstable in a 2-h chase (Fig. 2). Cleavage of SecA195 was not complete, since unproteolyzed full-length protein was also detected. The instability of the proteolytic fragment suggested that SecA function may have been perturbed by the proteolysis. We monitored translocation of OmpA precursor in a pulse-chase experiment which was done as described above, except that polyclonal antibodies against OmpA were used. When indicated, sodium azide was added to growing cultures 5 min prior to pulse-labeling at a final concentration of 3 mM. In

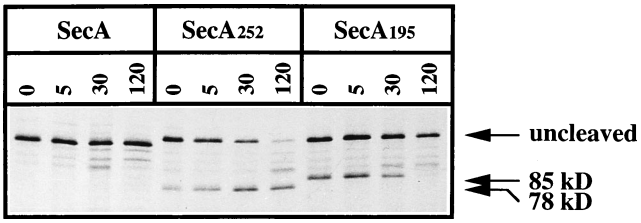

FIG. 2. Kinetics of SecA195 and SecA252 proteolysis. Cells of strain MC4100 expressing either wild-type SecA, SecA195, or SecA252 from the chromosome and TEV protease from pGEX-2T were labeled with [^{35}S]methionine for 45 s (lane 0) or 45 s followed by chase periods of 5, 30, or 120 min (lanes 5, 30, and 120, respectively) with excess cold methionine. Cells were lysed and immunoprecipitated with antibodies to SecA. The positions of SecA and of the proteolytic fragments with sizes of 85 kDa (SecA195) and 78 kDa (SecA252) are indicated by arrows.

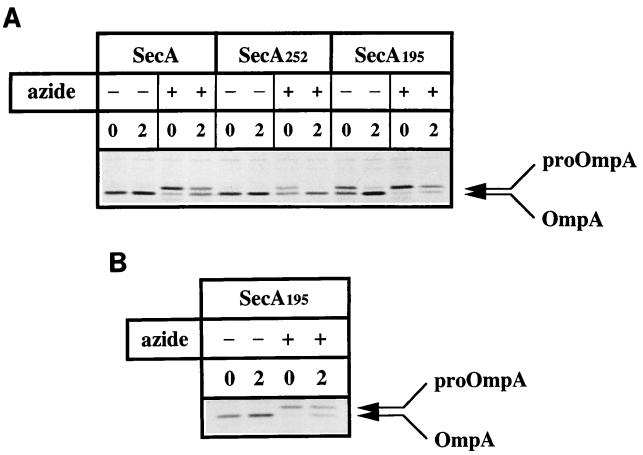

FIG. 3. Precursor accumulation in strains containing either proteolyzed or unproteolyzed SecA derivatives. (A) Cells of strain MC4100 expressing either wild-type SecA, SecA195, or SecA252 from the chromosome and TEV protease from pGEX-2T were labeled with [^{35}S]methionine for 45 s (lane 0) or 45 s followed by a 2-min (lane 2) chase period with excess cold methionine. Five minutes prior to labeling, cells were treated with 3 mM Na-azide (+) where indicated. Cells were lysed and immunoprecipitated with antibodies to OmpA. The positions of OmpA precursor (proOmpA) and of the mature form of OmpA are indicated. (B) The effect of the TEV protease cleavage site insert in SecA195 on secretion of proOmpA was assayed as described for panel A, except that the cells did not express TEV protease.

the absence of sodium azide, a secretion defect was detected in cells containing proteolyzed SecA195 but not in cells expressing wild-type SecA and TEV protease (Fig. 3A). In the presence of sodium azide, even stronger secretion defects were observed. To exclude the possibility that the protease cleavage site insert caused the secretion defect, the experiment was repeated in the absence of TEV protease. No accumulation of OmpA precursor was detected (Fig. 3B).

Since the GST-TEV protease hybrid protein was expressed under *tac* promoter control, we determined the optimal protease concentration for SecA cleavage. Cleavage was best in the presence of 5 and 10 μM IPTG. Raising the IPTG concentration above 0.1 mM caused aggregation of the GST-TEV protein. No SecA cleavage was detected under these conditions.

Coexpression of *secA252* and TEV protease. The *secA252* derivative of strain MC4100 was transformed with pGEX-2T encoding GST-TEV protease. SecA252 was cleaved by TEV protease in vivo. Proteolytic fragments of the predicted molecular masses of 28 kDa (N terminus) and 78 kDa (C terminus) could be detected on a Western blot by monitoring the state of SecA during stationary phase (Fig. 4).

Since the strain coexpressing SecA252 and TEV protease did not show any loss in viability under the conditions tested, even when complete cleavage occurred under stationary-phase conditions, we speculated that SecA252 remains functional in the proteolyzed state. To estimate the kinetics of proteolytic cleavage, we performed a pulse-chase experiment. SecA252 was cleaved by TEV protease with a half-life of about 55 min (Fig. 2). In contrast to SecA195, most of the large proteolysis product of SecA252 was stable in a 2-h chase (Fig. 2), and no secretion defect was detected when OmpA precursor was used as a reporter protein (Fig. 3). The proteolytic fragment with a size of 28 kDa was detected only on Western blots but not after immunoprecipitation.

One explanation for the detected activity of SecA252 after proteolysis is that cleaved SecA252 may not disassemble. We

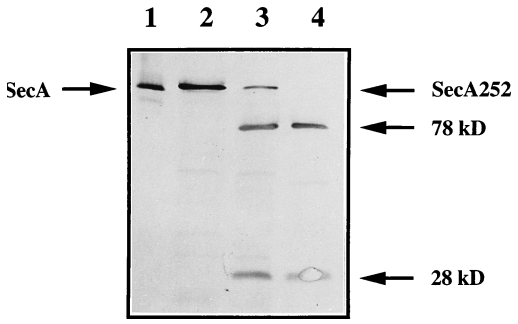

FIG. 4. Cleavage of SecA252 by TEV protease. To monitor the cleavage of SecA252 by TEV protease under stationary-phase conditions, a Western blot was prepared with cells of MC4100 expressing either wild-type SecA (lane 1) or SecA252 (lane 2) after overnight growth in Luria broth. The growth medium was without (lane 3) or supplemented with (lane 4) 10 μM IPTG to induce TEV protease. The positions of SecA and the proteolytic fragments with sizes of 28 and 78 kDa are indicated.

wanted to test whether functional SecA can be generated by expressing two SecA fragments in *trans*, corresponding to the proteolytic fragments generated from SecA252. We constructed two plasmids: pNT3, expressing the N-terminal 252 amino acids, and pCT3, expressing the C-terminal 663 amino acids of SecA. To generate plasmid pNT3, a 1,421-bp *Pst*I-*Bgl*II fragment of p*secA252* was subcloned into pACYC177 digested with *Bam*HI and *Pst*I. The *Bgl*II site is located within the sequence encoding the protease cleavage site. pCT3 was constructed by cloning the 2,115-bp *Mlu*I fragment of p*secA252*, which had been filled in with Klenow enzyme, into the *Eco*RI restriction site of pBAD18s (6), which had also been filled in with Klenow enzyme.

To assay whether SecA fragments expressed from pNT3 and pCT3 could assemble into a functional SecA dimer in the cell, these plasmids were transformed into strain MM52 [*secA*(Ts)]. The presence of pNT3 and pCT3, either alone or in combination, did not complement the *secA*(Ts) mutation, because transformants were unable to grow at 42°C.

Protease treatments are generally carried out in vitro or in spheroplasts of gram-negative bacteria. We wanted to extend the use of proteases for the in vivo analysis of cytoplasmic proteins by cleaving the target protein via a coexpressed specific protease. Ideally, the target protein should be the only substrate of the protease. We chose TEV protease, which recognizes specific sequences of seven amino acids and thus provided the desired specificity. This protease was used successfully in yeast cells to establish a genetic selection for the isolation of eucaryotic cDNAs encoding proteases which cleave within a defined amino acid sequence (18). We chose SecA, an essential protein involved in secretion, as the target protein.

We introduced a TEV protease cleavage site at two positions in SecA, i.e., after amino acids 195 and 252. Both SecA derivatives were cleaved in cells coexpressing TEV protease. After proteolytic cleavage, the function of SecA195 was significantly reduced, as was shown by the accumulation of OmpA precursor and the instability of the detected proteolytic SecA fragment. This novel approach could possibly be applied to inactivation of essential proteins in vivo and may serve as an alternative to conditional mutants and nonsense mutant dilution experiments.

Since TEV protease can only cleave surface sites, our results provide direct evidence for surface exposure of the TEV protease cleavage sites in the functional SecA dimer. Thus, in vivo proteolysis may be useful in probing for surface-accessible domains in proteins, for example, in structural studies and in protein engineering.

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