Tethering of the *Bacillus subtilis* $\sigma^E$ Proprotein to the Cell Membrane Is Necessary for Its Processing but Insufficient for Its Stabilization

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$\sigma^E$, a sporulation-specific transcription factor of *Bacillus subtilis*, is synthesized as an inactive proprotein with a 27-amino acid extension at its amino terminus. This “pro” sequence is removed by a developmentally regulated protease, but when present, it blocks $\sigma^E$ activity, tethers $\sigma^E$ to the bacterium’s cytoplasmic membrane, and promotes $\sigma^E$ stability. To investigate whether pro-$\sigma^E$ processing and/or stabilization are tied to membrane sequestration, we used fluorescent protein fusions to examine the membrane binding of SigE variants. The results are consistent with membrane association as a prerequisite for pro-$\sigma^E$ processing but not as a sufficient cause for the proprotein’s stability.

During the process of endospore formation (reviewed in references 16, 20, and 28), *Bacillus subtilis* undergoes an asymmetric cell division that yields two dissimilarly sized compartments within a common cell wall. The smaller compartment (forespore) develops into the endospore with the larger compartment (mother cell), aiding in the development of the forespore and then lysing to release the mature spore. The program of gene expression in each of these compartments is distinct and determined by the sequential appearance of unique RNA polymerase $\sigma$ factors, namely, $\sigma^E$ and then $\sigma^K$ in the mother cell and $\sigma^F$ followed by $\sigma^G$ in the forespore (16, 28). The $\sigma$ factors directing the initial programs of transcription in the mother cell and forespore ($\sigma^E$ and $\sigma^K$, respectively) are synthesized at the onset of sporulation but are kept inactive until the septation event establishes the two compartments. The mechanisms for blocking $\sigma^E$ and $\sigma^K$ activities are unique to each $\sigma$ factor. $\sigma^E$ is held inactive in a complex with an anti-$\sigma^E$ inhibitor (SpoIAB) (1, 4, 21), while $\sigma^K$ is synthesized as an inactive proprotein (pro-$\sigma^K$) (17, 27). $\sigma^K$ becomes active when an additional protein, SpoIIAA, binds to the SpoIAB inhibitor and allows $\sigma^E$ to be freed (reviewed in references 16 and 28).

Activation of $\sigma^E$ occurs by the removal of 27 amino acids from its amino terminus (16). The protease responsible for this activation, SpoIIGA, is coexpressed with pro-$\sigma^E$ but is inactive until the septum forms (10, 13, 15, 17, 19). Both pro-$\sigma^E$ and SpoIIGA are membrane bound and may be enriched at the septum (5, 7, 11, 18, 23). Processing of pro-$\sigma^E$ occurs when SpoIIR, a protein synthesized in the forespore and possibly secreted across the septal membrane, triggers SpoIIGA to cleave the pro sequence from pro-$\sigma^E$ and release $\sigma^E$ into the mother cell cytosol (8, 13, 18, 19). Although pro-$\sigma^E$ and SpoIIGA are synthesized prior to the division of the sporulating cell into forespore and mother cell compartments, $\sigma^E$ activity is restricted to the mother cell. This is likely to be due to the accumulation of $\sigma^E$ in that compartment as a consequence of its degradation in the forespore (12, 26) and its ongoing synthesis in the mother cell (2, 6).

The “pro” sequence of $\sigma^E$ has several interesting properties. It tethers $\sigma^E$ to the cytoplasmic membrane, serves as a target for the processing reaction, stabilizes the proprotein, and silences its activity as a transcription factor (7, 11, 17, 22). It could be envisioned that all of these features are interrelated, with membrane sequestration serving as the basis for all four pro sequence activities. In such a model, transfer to the membrane not only carries the proprotein to the site of SpoIIGA processing, but also places it in an environment which both protects it from degradation and removes it from possible RNA polymerase interactions. In a previous study, a number of mutations within the pro sequence region were constructed. Included in this collection were both a series of amino terminal deletions and point mutations (22). The mutations (Fig. 1) had been analyzed for their effect on SigE activity, stability, and processability. It was determined that deleting up to 10 amino acids from the SigE amino terminus (SigE388.5) (Fig. 1) did not alter its measurable activities, while deleting 16 amino acids (SigE335) resulted in a protein that was no longer processable but was active without processing. A similar phenotype was seen with a mutant with only six amino acids at its amino terminus. Removal of the entire pro sequence coding region (SigE78) yielded a sigE allele whose product failed to accumulate in *B. subtilis*. Thus, 6 amino acids of the pro sequence are sufficient for the protein to accumulate at high enough levels for detection by Western blotting, while 11 amino acids of the pro sequence are inadequate for processing to occur. The mutant sigE collection also contained a missense mutation (sigE25EK) which encoded an unprocessable proprotein. The residue altered in this allele, glu25, apparently defines a site required for recognition by the SpoIIGA processing enzyme. The sigE25EK mutation was found to be suppressible by a compensating mutation in spoIIGA (24).

The pro sequence has the potential for forming an $\alpha$ helical structure with positively charged and hydrophobic faces (Fig. 1 A and B, respectively). To test whether the orientation of these
faces is important for the pro sequence’s activity, we had inserted the coding sequence for one and then two additional amino acids into the sigE sequence (Fig. 1, SigE T1 and T2). This exercise misaligned and then realigned the charged and hydrophobic faces of the helix around residue 11 of the pro sequence. The insertion of one amino acid (Fig. 1, T1) reduced pro-αH abundance but did not alter its processing, while the addition of three amino acids (Fig. 1, T1 and T2), which should have realigned the faces of the helix, resulted in a SigE variant which was degraded and displayed no αH activity in vivo (22).

In light of the pro sequence’s ability to tether SigE to the B. subtilis membrane, we now use this mutant collection to ask whether there is a correlation between membrane binding and αH’s ability to be processed or stabilized. Given that the pro-αH processing enzyme (SpoIIGA) is thought to be membrane bound, a coincidence of pro-αH’s ability to bind to membranes and its processing might be anticipated; however, a possible correlation between stability and membrane association is less clear. To test the membrane binding capacity of our various sigE mutants, we created in-frame fusions of the sigE variants to green fluorescent protein (GFP) and examined their subcellular localization by fluorescence microscopy (11). DNA segments encoding the pro-SigE variants were joined to GFP at a site in sigE that corresponds to amino acid 55 of wild-type sigE protein. The integrating plasmid vector included an additional 500-bp fragment extending from nucleotide 450 of the 927-nucleotide spoIIGA gene to the intergenic region between spoIIGA and sigE. The placement of this additional region of homology upstream of the sigE:gfp fusions facilitated their insertion into the B. subtilis chromosome at spoIIG. The integration event keeps spoIIGA intact but places sigE::gfp as the expressed sigE allele. This separates the wild-type sigE from its promoter and renders the transformants SigE− and Spo−. These Bacillus strains, lacking αH, are blocked at stage II of sporulation and display a terminal disporic phenotype (i.e., sporulation septa are present at both poles of the cell) (25). In order to block potential processing of the fusion proteins and the release of GFP from the membrane, the processing-essential spoIIAC gene was disrupted in these strains. This involved transforming them with chromosomal DNA from a B. subtilis strain (EUR9030) carrying an antibiotic resistance cassette within spoIIAC (spoIIAC::erm) (14). Figure 2 illustrates fluorescence micrographs of cultures harvested at T4 of sporulation. Strains that carry a full-length pro sequence (A, B, C, and D), regardless of whether the sequence allowed the formation of a stable pro-αH (i.e., SigE T1 and T2 in Fig. 2D) or recognition for processing (Fig. 2B), displayed membrane-associated fluorescence, most notably at the polar septa. Membrane localization was also seen in the sigE deletion mutant (sigE388.5 in Fig. 2E) whose pro sequence could be processed but not in the deletion variant (sigE335 in Fig. 2F) whose product could not be processed.

In an attempt to determine whether the failure of SigE335 to be processed is a consequence of its cytoplasmic location or the loss of an explicit sequence required for recognition by the SpoIIGA, we created an artificial extension on the SigE335 amino terminus that mimicked the charge and hydrophobicity pattern of pro-αH, but we generated this structure by using alternative amino acid residues (SigE336 in Fig. 1). This was accomplished by a PCR technique in which the sigE sequence was amplified using an upstream oligonucleotide that included the coding sequence for the amino acid extension depicted in Fig. 1 (SigE366). The oligonucleotide also contained a restriction endonuclease site (BamHI) which would allow the amplified fragment to be joined in frame to the initiating methionine codon and ribosomal binding site of sigE. These elements were contained on an integrating plasmid vector that we had previously created to exchange pro sequence elements (3). The PCR amplification used downstream oligonucleotides that terminated the sigE fragment with an appropriate restriction endonuclease site either at sigE’s 55th codon, for fusion to GFP, or at the end of the sigE open reading frame, for synthesis of a full-length variant protein. The sigE366:gfp fusion was integrated into B. subtilis, as was done for the previous gfp fusions, and was examined by fluorescence microscopy. As illustrated in Fig. 2G, reestablishment of the pro-like sequence redirected the GFP fusion protein to the B. subtilis membrane. To examine the consequences of this membrane localization, the intact sigE336 allele was transformed into B. subtilis on an integrating plasmid similar to that which was used to transfer the gfp fusions. As in the previous plasmid integrations, the absence of a spoIIG promoter on the plasmid results in only one of the two sigE alleles (wild-type or sigE336) being expressed following Campbell-like integration of the plasmid into the B. subtilis chromosome. The sigE336 mutation is approximately midway in the Bacillus DNA fragment cloned within the plasmid. Thus, assuming random recombination along this sequence, approximately half of the transformants would be expected to contain sigE336 as the expressed sigE allele. All of the transformants formed brown colonies, as is typical of Spo+ B. subtilis on Difco sporulation medium (DSM) plates, suggesting that the

FIG. 1. Structure of SigE pro sequences. The helical model in the upper left portion of the figure represents a putative secondary structure for the wild-type SigE pro sequence from amino acids 3 to 21. The model illustrates the potential basic (A) and hydrophobic (B) faces of the predicted α helix. To the right and below the helix model are the amino acid sequences of the pro sequence of wild-type Bacillus subtilis sigE (SigE) as well as those of sigE deletion mutations (SigE78, -335, and -388.5), insertion mutations (for T1, an R placed between amino acids L11 and W12; for T2, an LA placed between amino acids H10 and L11), and a missense mutation (25EK). SigE336 is SigE335 with an additional region of amino acids which, when changed to lysine, prevents processing.

This exercise misaligned and then realigned the charged and hydrophobic faces of the helix around residue 11 of the pro sequence. The highlighted glutamate residue (no. 25) represents an amino acid which, when changed to lysine, prevents processing.

In order to block potential processing of the fusion proteins and the release of GFP from the membrane, the processing-essential spoIIAC gene was disrupted in these strains. This involved transforming them with chromosomal DNA from a B. subtilis strain (EUR9030) carrying an antibiotic resistance cassette within spoIIAC (spoIIAC::erm) (14). Figure 2 illustrates fluorescence micrographs of cultures harvested at T4 of sporulation. Strains that carry a full-length pro sequence (A, B, C, and D), regardless of whether the sequence allowed the formation of a stable pro-αH (i.e., SigE T1 and T2 in Fig. 2D) or recognition for processing (Fig. 2B), displayed membrane-associated fluorescence, most notably at the polar septa. Membrane localization was also seen in the sigE deletion mutant (sigE388.5 in Fig. 2E) whose pro sequence could be processed but not in the deletion variant (sigE335 in Fig. 2F) whose product could not be processed.

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The sigE336 allele encodes a functional SigE protein. To identify sigE336-expressing strains, chromosomal DNA was extracted from several transformants and screened by PCR for the presence of the sigE336 allele, with its unique BamHI site, as the sigE gene upstream of the vector and coupled to the spoIIG promoter. Two sigE336-expressing strains identified by this means were screened for their sporulation efficiency based on resistance to heat and chloroform (24). After an overnight incubation in DSM, less than 0.2% of the sigE335 cells were resistant to 80°C and chloroform, while 62% of the wild-type B. subtilis strain SMY CFU survived this treatment. Both sigE336-expressing strains displayed resistances (54 and 67%) similar to that of the wild-type strain.

The wild-type sporulation frequency of sigE336-expressing strains demonstrates that SigE336’s artificial extension and reassociation with the membrane had corrected SigE335’s deficiency. A likely mechanism for this phenomenon is the SigE336 pro protein’s ability to be processed now. We examined this possibility by allowing wild-type B. subtilis, as well as sigE335- and sigE336-expressing strains, to sporulate in DSM and by examining SigE accumulation and processing by Western blot analyses. As seen in Fig. 3, in contrast to sigE335, which encodes a protein of intermediate size that is not converted into mature σE, the sigE336-expressing strain displays a pattern of SigE accumulation and processing that is similar to the pro-σE/σE profile seen in the wild-type strain. To verify that the apparent processing of SigE336 was due to the normal processing reaction rather than fortuitous proteolysis and to determine the extent to which σE activity of this strain mimicked that of its parent strain, a σE-dependent reporter system

FIG. 2. Localization of SigE::GFP chimeras in sporulating B. subtilis. B. subtilis containing GFP fusions to wild-type SigE (A), SigE25EK (B), SigET1 (C), SigET1T2 (D), SigE388.5 (E), SigE335 (F), or SigE336 (G) under the control of the B. subtilis spoIIG promoter were grown in DSM until 4 h after the end of exponential growth and examined by phase-contrast (upper) and fluorescence (lower) microscopy, as described previously (11).

FIG. 3. Western blot analyses of SigE mutants. Cultures of wild-type B. subtilis strain SMY and variants of this strain expressing sigE335 or sigE336 were grown in DSM at 37°C and harvested at 1.5, 3, 4.5, and 6.0 h (lanes 1 to 4, respectively) after the end of exponential growth. Samples were processed and assayed by Western blot as described previously (23). The arrows indicate the pro-σE and σE bands in the SMY analysis.
membrane binding and protein stabilization. The SigE pro sequence is still able to target GFP to the cell membrane, while a variant (SigE335) whose truncated pro sequence fails to sequester a GFP fusion protein to the cell membrane could be readily detected by Western blotting. Apparently, membrane sequestration is insufficient for pro-σΔ stability.

It is also probable that the pro sequence’s effect on restricting σΔ activity is not solely a function of its membrane binding. We have noted in other work (McBride and Haldenwang, unpublished) that even though SigE335 is not membrane bound, its partial pro sequence reduces its activity to approximately 25% of that of wild-type σΔ in vivo. It has been shown by others that the pro sequence of σK, another membrane associating pro-σ factor, directly affects σKΔ in vitro associations with RNA polymerase and transcriptional activity (9). Thus, although the pro sequence’s ability to tether pro-σΔ to the cell membrane appears to be a prerequisite for σΔ processing, this feature is unlikely to be the basis of the proprotein’s stability and inactivity.

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REFERENCES


21. Min, K.-T., C. M. Hilditch, B. Diederich, J. Errington, and M. D. Yudkin. 1993. σΔ, the first compartment-specific σ factor of B. subtilis, is regulated by an anti-σ factor that is also a protein kinase. Cell 74:735–742.


