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SpoIVB and CtpB Are Both Forespore Signals in the Activation of the Sporulation Transcription Factor σ^K in *Bacillus subtilis*▽†

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The proteolytic activation of the mother cell transcription factor pro-σ^K is controlled by a signal transduction pathway during sporulation in the bacterium *Bacillus subtilis*. The pro-σ^K processing enzyme SpoIVFB, a membrane-embedded metalloprotease, is held inactive by two other integral membrane proteins, SpoIVFA and BofA, in the mother cell membrane that surrounds the forespore. Two signaling serine proteases, SpoIVB and CtpB, trigger pro-σ^K processing by cleaving the regulatory protein SpoIVFA. The SpoIVB signal is absolutely required to activate pro-σ^K processing and is derived from the forespore compartment. CtpB is necessary for the proper timing of σ^K activation and was thought to be a mother cell signal. Here, we show that the *ctpB* gene is expressed in both the mother cell and forespore compartments but that synthesis in the forespore under the control of σ^G is both necessary and sufficient for the proper timing of pro-σ^K processing. We further show that SpoIVB cleaves CtpB in vitro and in vivo but that this cleavage does not appear to be necessary for CtpB activation. Thus, both signaling proteins are made in the forespore and independently target the same regulatory protein.

Sporulation in the gram-positive bacterium *Bacillus subtilis* is a highly coordinated process involving several pathways of intercellular signaling. Upon a commitment to sporulate, the developing cell divides asymmetrically, generating two cells of unequal size and dissimilar developmental fates: a small cell (the prospective spore), referred to as the forespore, and a large cell called the mother cell (11, 14, 19, 27). Initially, these two cells lie side by side, but as sporulation progresses, the mother cell engulfs the forespore, generating a cell within a cell (Fig. 1A). As a result of engulfment, the forespore is surrounded by two membranes: its own, referred to as the inner forespore membrane, and one derived from the mother cell called the outer forespore membrane. Throughout the course of this developmental process, the mother cell and forespore communicate with each other to ensure that gene expression in one cell is coordinated with gene expression in the other. Four sporulation-specific RNA polymerase sigma factors are activated in a spatially and temporally restricted manner. Shortly after polar division, the transcription factor σ^F is activated in the forespore. σ^F is responsible for the activation of σ^E in the mother cell. At a later stage, σ^E is required for the activation of σ^G in the engulfed forespore. Finally, σ^G is required for the activation of σ^K in the mother cell. While the overall scheme of these signal transduction pathways has been elucidated, many of the mechanistic details concerning how information is transduced across the membrane remain unknown.

The late-acting mother cell transcription factor σ^K is synthesized as an inactive membrane-associated precursor protein (pro-σ^K) (15, 34). A signaling protein synthesized in the forespore under the control of σ^G triggers the proteolytic processing of pro-σ^K to its mature and active form (Fig. 1A) (6). The protease responsible for the activation of σ^K is the mother cell membrane protein SpoIVFB (7, 16, 21, 35). SpoIVFB is a founding member of a family of membrane-embedded metalloproteases involved in regulated intramembrane proteolysis (1, 2, 17, 23, 33). It is held inactive by two other mother cell membrane proteins, SpoIVFA and BofA (7, 22). These three proteins reside in a multimeric complex that localizes to the outer forespore membrane (20, 24). The signaling protein SpoIVB (not to be confused with the pro-σ^K processing enzyme SpoIVFB) is synthesized in the forespore and secreted into the space between the mother cell and forespore membranes (5). SpoIVB is a serine protease and triggers pro-σ^K processing by cleaving the extracellular domain of SpoIVFA at multiple sites (3, 9, 30). This cleavage is thought to cause a conformational change in the signaling complex that activates SpoIVFB-dependent pro-σ^K processing (3). A SpoIVFA mutant that cannot be cleaved by SpoIVB is delayed in pro-σ^K processing but not completely blocked, suggesting that SpoIVB has a second target other than SpoIVFA (3).

A second secreted serine protease, CtpB, is required for fine-tuning the timing of σ^K activation (18). In the absence of CtpB, pro-σ^K processing is delayed by approximately 30 min, and sporulation efficiency is reduced approximately twofold (3, 18). Expression profiling and promoter mapping suggest that CtpB is synthesized in both the mother cell under the control of σ^E (10, 18) and the forespore under the control of σ^G (26, 31). The CtpB signaling protease, like SpoIVB, targets the regulatory protein SpoIVFA (3). When SpoIVB is unable to cleave SpoIVFA, CtpB is capable of triggering pro-σ^K processing albeit after some delay. Importantly, a SpoIVFA mutant that cannot be cleaved by either CtpB or SpoIVB is completely blocked in σ^K activation, and sporulation efficiency is reduced

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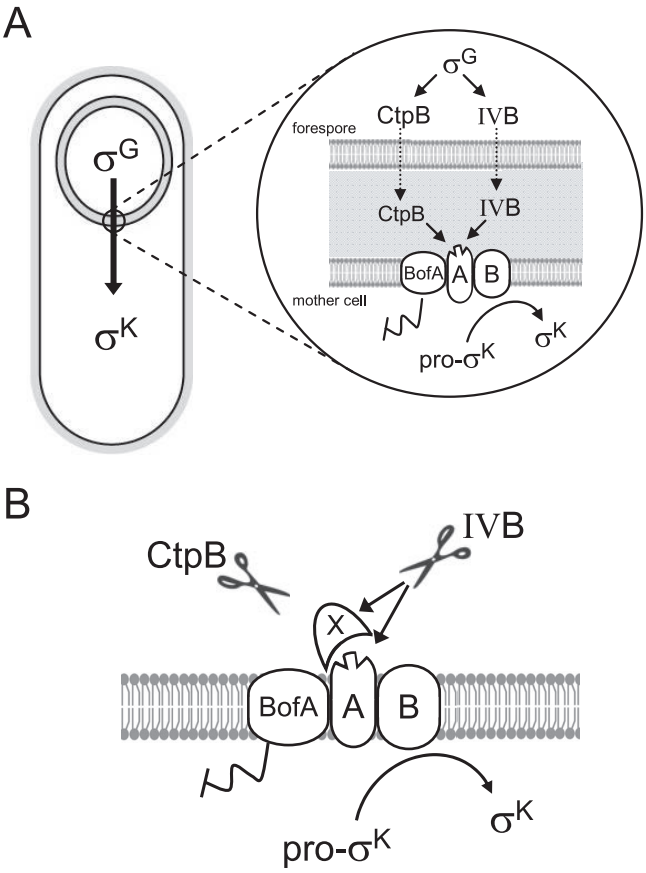

FIG. 1. SpoIVB (IVB) and CtpB are both forespore signals in the activation of the sporulation transcription factor σ^K. (A) After the completion of engulfment, a signal transduction pathway under the control of σ^G in the forespore activates σ^K in the mother cell. σ^K is synthesized as an inactive precursor protein (pro-σ^K). Proteolytic activation of pro-σ^K in the mother cell requires the membrane-embedded metalloprotease SpoIVFB (B). SpoIVFB is held inactive by the mother cell membrane proteins SpoIVFA (A) and BofA. The forespore-signaling serine proteases, SpoIVB and CtpB (scissors), are synthesized in the forespore under the control of σ^G and secreted into the space between the mother cell and forespore membranes. These two proteases trigger pro-σ^K processing by cleaving the extracellular domain of the regulatory protein SpoIVFA. SpoIVB is the principal signaling protease and is absolutely required to relieve the inhibition imposed on the processing enzyme SpoIVFB. CtpB is required for fine-tuning the timing of pro-σ^K processing. (B) The data described in this paper suggest that SpoIVB has another target in the activation of σ^K. This putative target (X) prevents CtpB (and perhaps SpoIVB) from gaining access to SpoIVFA. SpoIVB must first cleave X before CtpB can cleave SpoIVFA.

50-fold (3). During sporulation, CtpB is cleaved in a SpoIVB-dependent manner, suggesting that CtpB could be the second target of SpoIVB (3). Since SpoIVB is absolutely essential for pro-σ^K processing, we previously hypothesized that SpoIVB triggers pro-σ^K processing directly by cleaving SpoIVFA and indirectly by cleaving and activating CtpB (3).

In this report, we further characterize the CtpB signaling protease. We show that CtpB is synthesized in both the mother cell and forespore compartments in firmation of expression profiling analysis. We further show that the synthesis of CtpB in the forespore is both necessary and sufficient for the proper timing of pro-σ^K processing. In addition, we demonstrate that SpoIVB cleaves CtpB at two closely spaced sites in vitro. Finally, we show that a CtpB mutant that cannot be cleaved by SpoIVB in vitro does not impair the timing of pro-σ^K processing during sporulation. The simplest interpretation of these data is that SpoIVB-dependent cleavage of CtpB is not required to activate CtpB protease activity. Altogether, these results indicate that CtpB and SpoIVB are both forespore signals and that both proteases act in parallel to trigger pro-σ^K processing. These data further suggest that the SpoIVB signaling protease must have yet another target other than the regulatory protein SpoIVFA.

MATERIALS AND METHODS

General methods. All *B. subtilis* strains were derived from the prototrophic strain PY79 (32). *Escherichia coli* strains used were DH5α and BL21(DE3)(pLysS). Sporulation was induced by resuspension at 37°C according the Sterlini-Mandelstam method (13). Purification of recombinant CtpB and the CtpB mutant was performed as described previously (3). Tables of strains, plasmids, and oligonucleotide primers used in this study are available in the supplemental material.

Whole-cell extract preparation and immunoblot analysis. At the indicated times after the initiation of sporulation (by resuspension), the optical density at 600 nm was measured (for equivalent loading), and samples (1.0 ml) were collected by centrifugation. Whole-cell extracts were prepared by the resuspension of cell pellets in 50 μl lysis buffer (20 mM Tris [pH 7], 10 mM EDTA, 1 mg/ml lysozyme, 10 μg/ml DNase I, 100 μg/ml RNase A, 1 mM phenylmethylsulfonyl fluoride) and incubation at 37°C for 10 min followed by the addition of 50 μl sodium dodecyl sulfate (SDS) sample buffer (0.25 M Tris [pH 6.8], 6% SDS, 10 mM EDTA, 20% glycerol) containing 10% 2-mercaptoethanol. Samples were heated for 5 min at 80°C prior to loading. The equivalent of ~75 μl of cells was analyzed per time point. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 12% polyacrylamide gels, electroblotted onto a polyvinylidene difluoride membrane (Perkin-Elmer), and blocked in 5% nonfat milk in phosphate-buffered saline–0.5% Tween 20. The blocked membrane was probed with affinity-purified anti-SpoIVB (3), anti-σ^K (20), anti-SpoIVFA (20), or anti-CtpB (3) antibodies. Primary antibodies were diluted 1:10,000 into 3% bovine serum albumin in phosphate-buffered saline–0.05% Tween 20. Primary antibodies were detected using horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad) with Western Lightning substrate (Perkin-Elmer) according to the manufacturer's instructions.

Plasmid construction. pNC114 [Δ*ss-ctpB*(*A36R,A40R*)-His_6] (Δ*ss* indicates that the putative signal sequence in *ctpB* has been removed) was generated by site-directed mutagenesis using oligonucleotide primer oNC77 and template DNA pDR211 (3).

pNC118 [*yycR*::*ctpB* (*phleo*)] was created in a two-way ligation with a BamHI-EcoRI fragment from pQP169 (18) containing the *ctpB* gene and pNS042 cut with BamHI and EcoRI. pNS042 is an ectopic integration vector for double-crossover insertions into the nonessential *yycR* chromosomal locus with the phleomycin resistance gene (N. Sullivan and D. Z. Rudner, unpublished data).

pNC121 [*yycR*::*ctpB*(*A36R,A40R*) (*phleo*)] was generated by site-directed mutagenesis using oligonucleotide primer oNC77 and template DNA pNC118.

pNC126 [*yycR*::P_{ctpB}-*ctpB* (*phleo*)] was created in a three-way ligation with an EcoRI-HindIII PCR product containing the *ctpB* promoter (oligonucleotide primers oDR425 and oDR426 and template DNA pNC118), a HindIII-BamHI PCR product containing the *ctpB* gene with an optimized ribosome binding site (RBS) (29) (oligonucleotide primers oDR427 and oDR428 and genomic DNA as a template), and pNS042 cut with EcoRI and BamHI. pNC128 [*yycR*::P_{sspB}-*ctpB* (*phleo*)] was generated in a three-way ligation with an EcoRI-HindIII fragment from pDT98 (T. Doan and D. Z. Rudner, unpublished data) containing the *sspB* promoter, a HindIII-BamHI PCR product containing the *ctpB* gene with an optimized RBS (oligonucleotide primers oDR427 and oDR428 and genomic DNA as a template), and pNS042 cut with EcoRI and BamHI.

pNC129 [*yycR*::P_{spoIID}-*ctpB* (*phleo*)] was generated in a three-way ligation with an EcoRI-HindIII fragment from pDR78 (24) containing the *spoIID* promoter, a HindIII-BamHI PCR product containing the *ctpB* gene with an optimized RBS (oligonucleotide primers oDR427 and oDR428 and genomic DNA as a template), and pNS042 cut with EcoRI and BamHI.

pNC136 [*amyE*::P_{ctpB}-yfp_{JF} (*spec*)] was generated in a two-way ligation with an

EcoRI-HindIII fragment from pNC126 containing the *ctpB* promoter and pKM022 cut with EcoRI and HindIII. pKM022 is derived from pLD30 (12) and contains the *yfp* gene from the jellyfish *Aequorea victoria* (yfp_{JF}) (K. Marquis and D. Z. Rudner, unpublished data). The *ctpB* promoter fragment was 400 bp and ended 13 bases before the *ctpB* start codon.

pNC153 [*amyE*::P_{sspB}-yfp_{JF} (*spec*)] was created in a two-way ligation with an EcoRI-HindIII fragment from pDT98 containing the *sspB* promoter and pKM022 cut with EcoRI and HindIII.

pNC171 [*yycR*::P_{spoIVF}-*ctpB* (*phleo*)] was created in a three-way ligation with an EcoRI-HindIII PCR product containing the *spoIVF* promoter (oligonucleotide primers oDR67 and oDR68 and template DNA pSC224) (7), a HindIII-BamHI PCR product containing the *ctpB* gene with the *spoIVFA* RBS (oligonucleotide primers oNC97 and oDR428 and genomic DNA as a template), and pNC126 cut with EcoRI and BamHI.

pNC177 [*yycR*::P_{ctpB}-*ctpB*(*A36R,A40R*) (*phleo*)] was created in a two-way ligation with a HindIII-BamHI PCR product containing the *ctpB*(*A36R,A40R*) gene with an optimized RBS (oligonucleotide primers oDR427 and oDR277 and template DNA pNC121) and pNC126 cut with HindIII and BamHI.

pKM012 [*amyE*::P_{spoIID}-yfp_{Bs} (*spec*)] *B. subtilis* codon-optimized *yfp* (yfp_{Bs}) (Marquis and Rudner, unpublished) containing an optimized RBS was subcloned into pDR78 between HindIII and BamHI.

Cleavage assay. Approximately 1.5 μg of purified CtpB-His_6 was mixed with 100 ng of purified SpoIVB (per time point) and incubated at room temperature. The reaction was stopped by the addition of 2× SDS-sample buffer and analyzed by SDS-PAGE followed by Coomassie staining. To determine the identity of the SpoIVB-dependent cleavage sites in CtpB, the cleavage reaction was loaded onto a C_{18} column and eluted with a linear gradient of acetonitrile. The elution products were monitored by mass spectrometry at the Taplin Biological Mass Spectrometry Facility at Harvard Medical School (Boston, MA).

Fluorescence microscopy. Fluorescence microscopy was performed with an Olympus BX61 microscope as previously described (24). Fluorescent signals were visualized with a phase-contrast objective UplanF1 100× and captured with a monochrome CoolSnapHQ digital camera (Photometrics) using Metamorph software, version 6.1 (Universal Imaging). Exposure times were typically 500 ms for yellow fluorescent protein (YFP) (excitation, 490 to 510 nm; emission, 520 to 550 nm). The lipophilic membrane dye TMA-DPH (Molecular Probes) was used at a final concentration of 0.05 mM, and exposure times were typically 200 ms (excitation, 340 to 380 nm; emission, 435 to 485 nm). Images were analyzed, adjusted, and cropped using Metamorph software.

RESULTS

CtpB is synthesized in both the mother cell and the forespore. Previous microarray analyses suggested that *ctpB* is controlled by σ^E in the mother cell and σ^G in the forespore (10, 26, 31). To investigate these results in vivo, we fused the *ctpB* promoter (P_{ctpB}) to the gene encoding YFP and monitored compartment-specific fluorescence during sporulation. As controls for this experiment, we analyzed strains harboring a mother cell-specific promoter controlled by σ^E (P_{spoIID}) or a forespore-specific promoter controlled by σ^G (P_{sspB}) (Fig. 2A) (19). σ^E-controlled gene expression initiates shortly after polar division and persists through the process of engulfment (27). By contrast, σ^G-controlled gene expression initiates in the forespore after the completion of engulfment. Consistent with the idea that CtpB is synthesized under the control of σ^E, we could detect mother cell-specific YFP fluorescence in cells that had begun the engulfment process (Fig. 2A), and no fluorescence was detected in a σ^E mutant (data not shown). Upon the completion of engulfment, YFP fluorescence was also observed in the forespore compartment, suggesting that CtpB is also synthesized under the control of σ^G (Fig. 2A). To confirm that the *ctpB* promoter was indeed recognized by σ^G, we monitored YFP fluorescence in a σ^G mutant. Under these conditions, only mother cell fluorescence (under σ^E control) was detected (Fig. 2A).

To confirm that the CtpB protein is synthesized in both

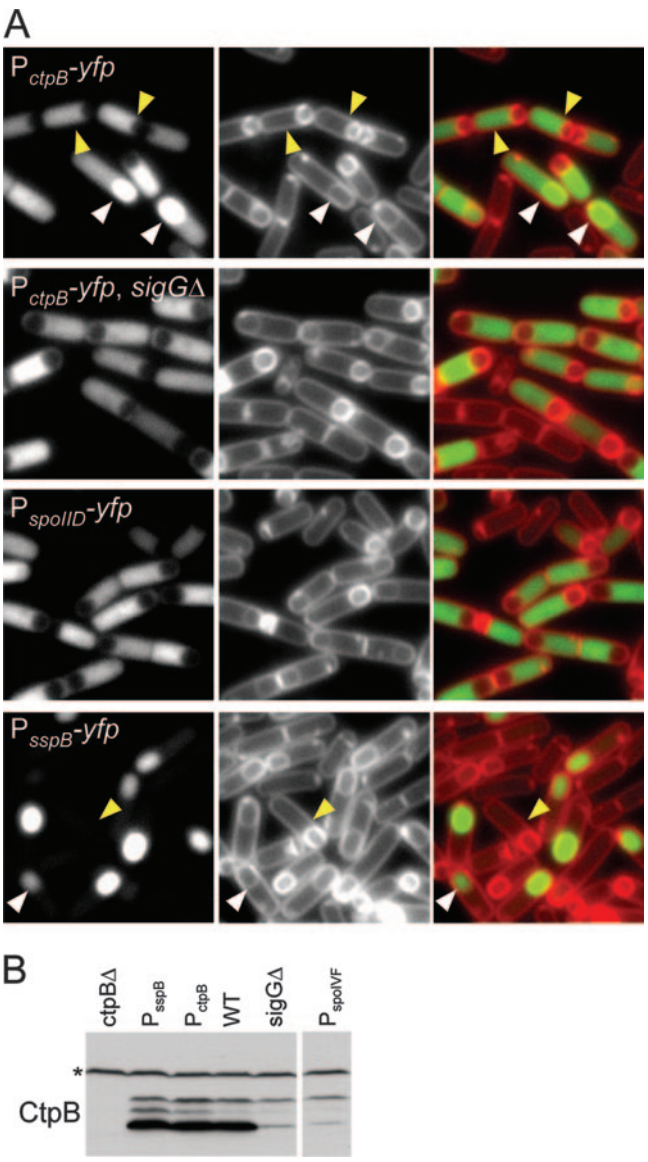

FIG. 2. CtpB is synthesized in both the mother cell under the control of σ^E and in the forespore under the control of σ^G. (A) Fluorescence microscopy of *B. subtilis* sporulating cells at hour 2.25. Compartment-specific gene expression was visualized using the *ctpB* promoter (P_{ctpB}) fused to the gene encoding yellow fluorescent protein (*yfp*) in the wild type (BN986) and in a σ^G mutant (BNC1014). For comparison, strains harboring *yfp* fusions to the mother cell-specific promoter P_{spoIID} controlled by σ^E (BKM34) or the forespore-specific promoter P_{sspB} controlled by σ^G (BNC1043) are shown. The membranes from the same fields were visualized using the fluorescent dye TMA-DPH. Examples of septated and engulfing cells (in which σ^G has not yet been activated) and a fully engulfed forespore (in which σ^G has been activated) are indicated with white and yellow carets, respectively. (B) Immunoblot analysis of whole-cell lysates from sporulating cells at hour 4. Strains analyzed were the wild type (WT) (PY79), a *ctpB* mutant (ctpBΔ) (QPB161), a σ^G mutant (sigGΔ) (BDR452), and a *ctpB* null mutant harboring the *ctpB* gene fused to the indicated promoters (P_{ctpB}, BNC978; P_{sspB}, BNC982; P_{spoIVF}, BNC1158) (see Materials and Methods). CtpB appears as three forms corresponding to the full-length protein (upper band), an autoproteolytic product (middle band), and a SpoIVB-dependent cleavage product (lower band) (3). A nonspecific band (asterisk) that cross-reacts with the anti-CtpB antibodies is indicated. The immunoblot was from the same gel (and the same autoradiography film), but control lanes were removed for clarity.

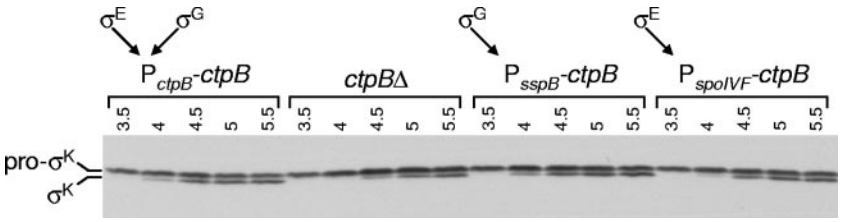

FIG. 3. Synthesis of CtpB in the forespore is necessary and sufficient for the proper timing of pro-σ^K processing. Immunoblot analysis of whole-cell lysates from sporulating cells. Strains analyzed were a *ctpB* null strain (BNC738) and the same strain harboring the indicated promoter fusions to the *ctpB* gene (P_{ctpB}, BNC1045; P_{sspB}, BNC1047; P_{spoIVF}, BNC1204). All four strains contained a SpoIVFA mutant [SpoIVFA(S155R)] that delays pro-σ^K processing (3). Pro-σ^K processing was monitored by immunoblotting using anti-σ^K antibodies. Time (in hours) after the initiation of sporulation is indicated. The sigma factors that recognize the different promoters are shown.

compartments, we performed immunoblots with anti-CtpB antibodies. In wild-type sporulating cells, full-length CtpB is converted into two smaller products: one species is a result of autoproteolysis, and a smaller product is due to SpoIVB-dependent cleavage (Fig. 2B) (3, 18, 36). Consistent with the idea that the majority of CtpB comes from the forespore compartment, the level of CtpB was greatly reduced in the absence of σ^G (Fig. 2B). The small amount of protein present in this background was due to synthesis under the control of σ^E since no CtpB was detected in the absence of this mother cell transcription factor (data not shown). We note that the SpoIVB-dependent cleavage of CtpB still occurs in the absence of σ^G (Fig. 2B) due to low-level expression of SpoIVB under the control of the earlier-acting forespore transcription factor σ^F (8).

These results indicate that CtpB is synthesized both in the mother cell under the control of σ^E and in the forespore under the control of σ^G. Moreover, our data suggest that most of the protein resident in the space between the mother cell and forespore membranes is derived from the forespore. Previous experiments using a *lacZ* fusion to the *ctpB* promoter suggested that *ctpB* is under the control of σ^E and only weakly under σ^G control (18). We cannot explain this discrepancy, but based on fluorescence microscopy and the immunoblot analysis presented here, we conclude that CtpB is synthesized under the control of both transcription factors.

Forespore-produced CtpB is necessary and sufficient for proper timing of pro-σ^K processing. To determine in which compartment CtpB is needed to be made for the proper timing of pro-σ^K processing, we fused the *ctpB* gene to mother cell-specific (P_{spoIVF}) and forespore-specific (P_{sspB}) promoters (19). As shown in Fig. 2B, these promoter fusions closely matched the contribution of σ^E and σ^G to the total amount of CtpB protein (Fig. 2B and see Materials and Methods). In our strain background, the delay in pro-σ^K processing in the absence of CtpB is subtle and varies between 15 and 20 min (3). Therefore, to increase the phenotypic window in which to assess the contribution of forespore-produced and mother cell-produced CtpB, we used a strain that is more sensitive to the loss of CtpB. This strain contains a mutation in a SpoIVB-dependent cleavage site in the SpoIVFA protein (3). In this background, pro-σ^K processing is delayed by 30 min when CtpB is absent (Fig. 3). The promoter fusions were inserted into this mutant, and pro-σ^K processing was monitored during a sporulation time course. When CtpB was synthesized in the forespore under the control of σ^G (P_{sspB}), the timing of pro-σ^K processing was indistinguishable from that of the wild type (Fig. 3). However, when CtpB was made in the mother cell under the control of σ^E (P_{spoIVF}), pro-σ^K processing was delayed by approximately 30 min. Similar results were obtained in the strain background in which the SpoIVFA protein was intact (see Fig. S1 in the supplemental material). These experiments indicate that CtpB made in the forespore is both necessary and sufficient for the proper timing of pro-σ^K processing.

We wondered whether the requirement for forespore-produced CtpB could be explained by the higher levels of CtpB made in this compartment (Fig. 2B). To test this, we fused *ctpB* to a much stronger σ^E promoter (P_{spoIID}) and strengthened the RBS of the *ctpB* gene (see Materials and Methods). Under these conditions, we were able to achieve significantly higher levels of CtpB than those normally made under σ^E control from the P_{ctpB} promoter. With these high levels of mother cell-produced CtpB, the timing of pro-σ^K processing was similar to that of the wild type (see Fig. S2 in the supplemental material). From these experiments, we conclude that CtpB, like SpoIVB, is a forespore signal but that CtpB can act as a mother cell signal provided that it is artificially synthesized to high levels.

CtpB is cleaved by SpoIVB in vitro. We have shown previously that CtpB is cleaved during sporulation and that this cleavage is SpoIVB dependent (3). To determine whether CtpB is a substrate of the SpoIVB protease, we purified a CtpB-His_6 fusion protein synthesized in *E. coli*. Incubation of SpoIVB with the purified protein resulted in the efficient cleavage of CtpB to a size similar to what was observed during sporulation in *B. subtilis* (Fig. 4A). To identify the SpoIVB-dependent cleavage site, we separated the cleavage reaction by reverse-phase high-performance liquid chromatography and analyzed the eluate by mass spectrometry (see Materials and Methods). Two cleavage sites were identified near the amino terminus of CtpB (Fig. 4B). To generate an uncleavable mutant, we substituted the alanines at the P1′ positions with arginine residues (A36R and A40R). This double mutant completely blocked SpoIVB-dependent cleavage of CtpB in vitro (Fig. 4A).

CtpB can cleave itself in vivo (3, 36); however, we did not detect this autoproteolytic activity in our in vitro assay. This could be because the autocleavage occurs within the putative signal sequence, which we removed from our fusion protein.

SpoIVB-dependent cleavage of CtpB is not critical for efficient pro-σ^K processing. We previously hypothesized that SpoIVB-dependent cleavage of CtpB serves to activate CtpB

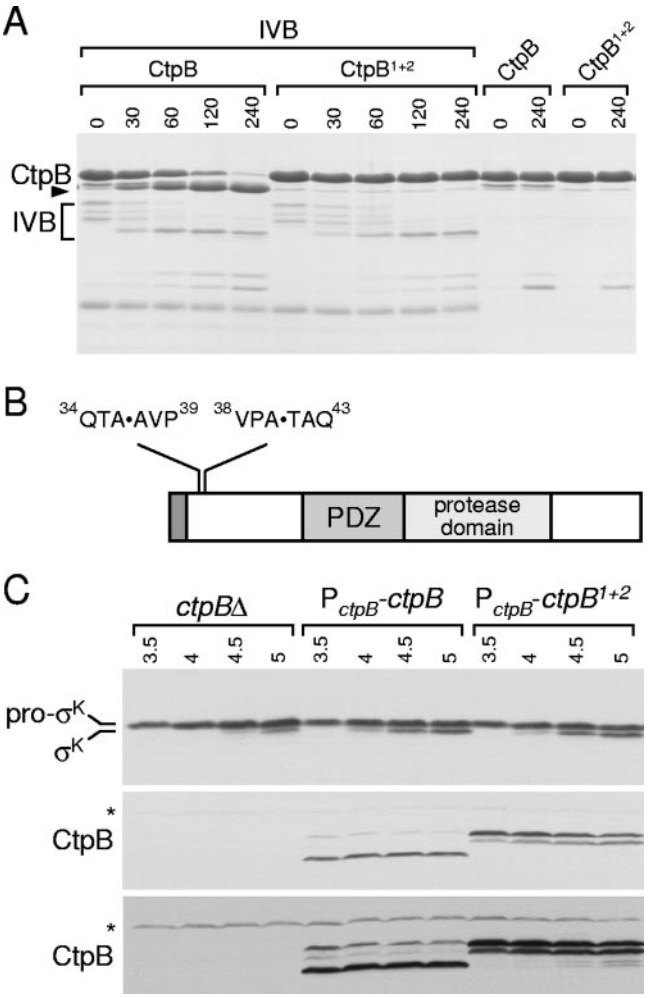

FIG. 4. SpoIVB-dependent cleavage of CtpB in vitro and in vivo. (A) SpoIVB cleaves CtpB in vitro. Purified CtpB and an uncleavable mutant ($CtpB^{1+2}$) were incubated with SpoIVB or buffer at room temperature for the indicated times (in minutes). Reaction mixtures were analyzed by SDS-PAGE and stained with Coomassie blue. The SpoIVB-dependent CtpB cleavage product is indicated (caret). The SpoIVB autoproteolytic cleavage products are also shown (bracket). (B) Schematic diagram of the full-length CtpB protein and the SpoIVB-dependent cleavage sites identified by mass spectrometry. CtpB contains a putative signal sequence, a PDZ domain, and a serine protease domain. The amino acids flanking the cleavage sites and their positions in the full-length CtpB protein are indicated. (C) SpoIVB-dependent cleavage of CtpB is not critical for efficient pro-σ^K processing. Immunoblot analysis of whole-cell lysates from sporulating cells is shown. Strains analyzed were a *ctpB* null strain (ctpBΔ) (BNC738), the same strain harboring the wild-type *ctpB* strain (P_{ctpB}-*ctpB*) (BNC1045), and the uncleavable *ctpB* mutant (P_{ctpB}-$ctpB^{1+2}$) (BNC1228). All three strains contained a SpoIVFA mutant [SpoIVFA(S155R)] that delays pro-σ^K processing (3). Time (in hours) after the initiation of sporulation is indicated. Pro-σ^K processing (top) and CtpB cleavage (middle and bottom) were monitored by immunoblotting. A nonspecific band (asterisk) that cross-reacts with the anti-CtpB antibodies is indicated. A short exposure (middle) and a long exposure (bottom) of the anti-CtpB immunoblot are shown to highlight the small amount of SpoIVB-dependent cleavage of CtpB in the $CtpB^{1+2}$ mutant.

in a manner analogous to that of serine protease cascades found in blood clotting (3). To test this hypothesis, we introduced the uncleavable *ctpB* mutant into *B. subtilis* and monitored pro-σ^K processing and CtpB cleavage during a sporulation time course. For these experiments, we used strains containing wild-type SpoIVFA or SpoIVFA mutants blocked at one [SpoIVFA(S155R)] or all of the SpoIVB-dependent cleavage sites (3). In all strain backgrounds tested, the timing of pro-σ^K processing was the same whether or not CtpB could be cleaved by SpoIVB (Fig. 4C and data not shown). Analysis of CtpB by immunoblot revealed that in the uncleavable CtpB mutant, the full-length protein and the autoproteolytic product accumulated (Fig. 4C). This result indicates that SpoIVB does indeed cleave CtpB in vivo. However, and importantly, this cleavage does not appear to be necessary for CtpB activity. We note that small amounts of SpoIVB-dependent cleavage products were detectable in our CtpB mutant (Fig. 4C, bottom), and it is formally possible that these products are the active species. Nonetheless, the simplest interpretation of these experiments is that CtpB does not require SpoIVB-dependent cleavage to be active in the pro-σ^K processing pathway.

DISCUSSION

Here, we report that the CtpB serine protease acts as a forespore signal for the activation of the mother cell transcription factor σ^K during sporulation in *B. subtilis*. Both SpoIVB and CtpB are synthesized under the control of σ^G and are translocated into the intermembrane space surrounding the forespore, where they trigger pro-σ^K processing by cleaving SpoIVFA. Although SpoIVB efficiently cleaves CtpB in vivo, this cleavage does not appear to be critical for CtpB activity and the proper timing of pro-σ^K processing.

The SpoIVB signal is absolutely required to trigger pro-σ^K processing (5). In its absence, processing is completely blocked. We have previously shown that a SpoIVFA mutant that cannot be cleaved by SpoIVB delays, but does not block, pro-σ^K processing (3). This result indicates that a second pathway under the control of SpoIVB can activate pro-σ^K processing. Since mature σ^K is undetectable in an uncleavable SpoIVFA mutant lacking CtpB, we proposed that the second target of SpoIVB is CtpB and that this cleavage would activate CtpB in a manner reminiscent of serine protease cascades. However, the data presented here suggest that this is not the case. Although we cannot rule out the possibility that the small amount of CtpB that is cleaved by SpoIVB in our uncleavable CtpB mutant is sufficient to trigger pro-σ^K processing or that SpoIVB can activate CtpB in a manner that does not depend on proteolysis, the simplest interpretation of our data is that CtpB is not the physiologically relevant second target of SpoIVB.

We envision two possible scenarios to explain why CtpB becomes essential for pro-σ^K processing when SpoIVB cannot cleave SpoIVFA. In the first model, we hypothesize that SpoIVB inefficiently cleaves SpoIVFA at a fifth site that we did not identify. On its own, this cleavage would not be sufficient to trigger pro-σ^K processing. However, in combination with the CtpB-dependent cleavage of SpoIVFA, pro-σ^K processing could occur. In the second model, we propose that SpoIVB has yet another target in this signaling pathway (Fig. 1B). This unidentified SpoIVB substrate would prevent CtpB (and perhaps SpoIVB) from gaining access to SpoIVFA. In this scenario, SpoIVB must cleave this protein *before* CtpB can cleave

SpoIVFA. When SpoIVB is unable to cleave SpoIVFA, SpoIVB would still be able to clear this protein out of the way, allowing CtpB to trigger pro-σ^K processing. Thus, in the absence of SpoIVB, CtpB is active but cannot access its substrate. We and others have found that SpoIVB is capable of cleaving the extracellular domain of the forespore membrane protein SpoIIQ (3, 4). However, SpoIIQ does not appear to be critical for the activation of σ^K since an uncleavable SpoIIQ mutant did affect the timing of pro-σ^K processing when SpoIVB was unable to cleave SpoIVFA (data not shown). The identification of this SpoIVB target remains the principal challenge for the future.

An unexpected and interesting finding to emerge from the analysis presented here is that CtpB is capable of acting as a mother cell signal when synthesized to artificially high levels. One of us proposed previously that during engulfment, integral membrane proteins are not inserted directly into the engulfing septal membrane from the mother cell side and instead achieve proper localization by insertion into the cytoplasmic membrane followed by diffusion to, and capture in, the outer forespore membrane (25). Since the machineries involved in membrane protein insertion and secretion are shared, it is not clear how mother cell-produced CtpB becomes localized in the intermembrane space. One possibility is that CtpB is a poor substrate for signal peptidase such that after it is translocated, it is not efficiently released from the membrane (28). If this is the case, then the membrane-tethered protease produced in the mother cell could diffuse into the intermembrane space and be cleaved and released by SpoIVB. In support of this idea, fractionation experiments indicate that full-length CtpB is membrane associated, while the SpoIVB-cleaved form is soluble (see Fig. S3 in the supplemental material). The prevalence of targeted secretion by "diffusion and cleavage," in which the protease that releases the secreted protein from the membrane is itself localized, awaits further investigation.

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