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A Branched Pathway Governing the Activation of a Developmental Transcription Factor by Regulated Intramembrane Proteolysis

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Summary

The proteolytic activation of the membrane-associated 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. We demonstrate that the signaling protease SpoIVB (IVB) triggers pro-σ^K processing by cleaving the extracellular domain of the SpoIVFA regulator at multiple sites. In vitro, these cleavages do not disrupt the interactions between SpoIVFA, SpoIVFB, and BofA, suggesting that IVB-dependent activation of the processing enzyme results from a conformational change in this complex. Our data further suggest that when IVB is unable to cleave SpoIVFA, it can still activate pro-σ^K processing through a second protease, CtpB. Finally, we demonstrate that CtpB, like IVB, triggers pro-σ^K processing by cleaving SpoIVFA. We propose that IVB regulates intramembrane proteolysis through two proteolytic pathways, both of which converge on the same regulator.

Introduction

Cells respond to their environment and to each other by transducing signals across their membranes to elicit transcriptional responses. One mechanism by which information is transduced across the membrane involves the cleavage of a protein within or adjacent to the lipid bilayer, resulting in the release of a membrane-anchored transcription factor (Brown et al., 2000). This type of signaling is broadly referred to as regulated intramembrane proteolysis (RIP). The intramembrane cleaving proteases (I-CLiPs) that mediate RIP are polytopic membrane proteins with catalytic-site motifs embedded within transmembrane segments (Weihofen and Martoglio, 2003). In virtually all examples of signal transduction involving RIP, regulation is exerted at the level of substrate processing or maturation (Wolfe and Kopan, 2004). The membrane substrate is not competent to be cleaved by the I-CLiP, as this proteolysis requires prior cleavage of the substrate on the extracellular (or luminal) side of the membrane. Thus, for example, in response to DSL ligands, the membrane-tethered transcription factor Notch is first cleaved by an ADAM protease on the extracellular side of the membrane, which leads to Presenilin-mediated cleavage within the membrane (Mumm and Kopan, 2000). Similarly, in response to a drop in cholesterol, the Sterol Response Element Binding Protein (SREBP) is first cleaved in the lumen of the Golgi by the Site-1 Protease, which results in intramembrane cleavage by the Site-2 Protease (S2P) (Nohturfft et al., 2000; Rawson et al., 1997). Here we are concerned with an example of RIP in which regulation of intramembrane proteolysis does not conform to the canonical pathway involving substrate maturation. The substrate, a bacterial transcription factor known as pro-σ^K, is synthesized without an extracellular domain and is fully competent to be cleaved by its cognate I-CLiP (SpoIVFB) (Resnekov and Losick, 1998; Zhou and Kroos, 2004). The I-CLiP activity of SpoIVFB (henceforth referred to as B, for simplicity) is negatively regulated, not by substrate inaccessibility as in the canonical pathway, but by two negative regulators (Cutting et al., 1990). In this work, we investigate how a signaling protease triggers intramembrane proteolysis in this exceptional example of RIP to transduce information across the membrane.

The σ^K transcription factor is responsible for gene expression during the developmental process of spore formation in *Bacillus subtilis*. In response to starvation, *B. subtilis* differentiates into a stress-resistant cell type known as a spore (Errington, 2003). A hallmark event in this process is the formation of an asymmetrically positioned septum, which generates two cells of unequal size: a larger mother cell and a small forespore (the prospective spore). These two cells initially lie side by side, but later in development the mother cell engulfs the forespore to create a cell within a cell (Figure 1A). At this late stage, the mother cell nurtures the forespore, packaging it in a protective protein coat while the forespore prepares for dormancy. Throughout this morphological process, the forespore and mother cell follow completely different programs of gene expression, but signal transduction pathways ensure that these developmental programs are temporally coordinated with each other.

σ^K directs late gene expression in the mother-cell compartment, including genes involved in the synthesis of the spore coat, and its activity is tightly regulated to ensure that transcription of these genes is kept in register with events occurring in the forespore (Cutting et al., 1990). Activation of σ^K is achieved by proteolytic processing of an inactive, membrane-associated precursor (pro-σ^K) and release into the mother-cell cytoplasm (Zhang et al., 1998). The conversion of pro-σ^K to σ^K, the consequence of RIP, requires the polytopic membrane protein B, which is synthesized in the mother-cell compartment and is likely to be the processing enzyme (Figure 1A) (Cutting et al., 1991b; Zhou and Kroos, 2004). B and the mammalian S2P involved in proteolytic activation of SREBP are founding members of a family of membrane-embedded Zn^{2+} metalloproteases (Akiyama et al., 2004; Rawson et al., 1997; Rudner et al., 1999; Yu and Kroos, 2000). Activation of intramembrane proteolysis by S2P and another well-characterized member of this family, RseP from *E. coli* (Alba et al., 2002; Kanehara

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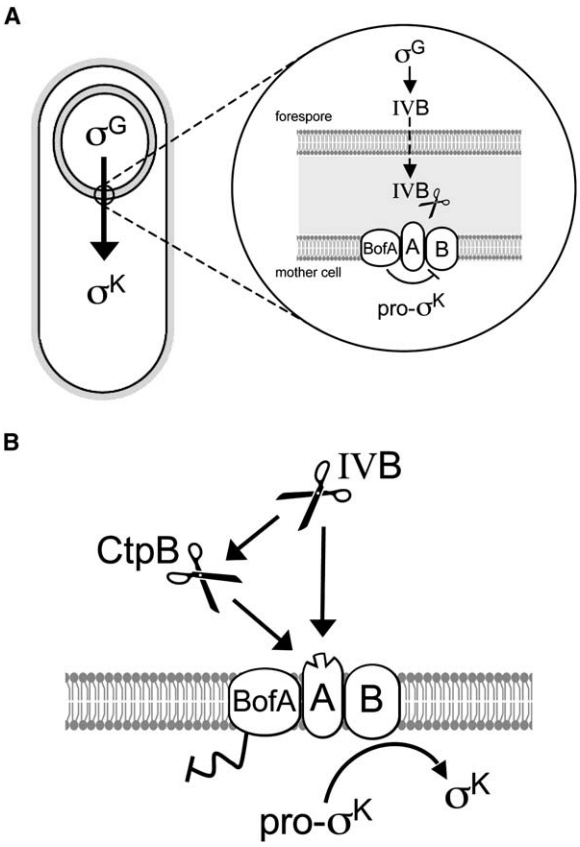

Figure 1. Schematic Representation of the Pro-σ^K Processing Pathway

(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 B. B is held inactive by two other membrane proteins, A and BofA. The forespore signaling protein IVB is synthesized under the control of σ^G and secreted into the space between the mother-cell and forespore membranes (shown in gray). IVB is a serine protease (scissors), and protease activity is required to relieve the inhibition imposed on the processing enzyme B by A and BofA.

(B) Two IVB-dependent pathways trigger pro-σ^K processing. IVB activates pro-σ^K processing directly by cleaving A at either of two sites and indirectly by activating the CtpB serine protease. Active CtpB, in turn, cleaves the extracellular domain of A.

et al., 2002), is triggered by the substrate maturation pathway described above. By contrast, B is negatively regulated by two integral membrane proteins, SpoIVFA (referred to as A) and BofA (Cutting et al., 1991b; Ricca et al., 1992). The processing enzyme and its two negative regulators reside in a multimeric membrane complex that localizes to the mother-cell membrane that surrounds the forespore (Figure 1A) (Resnekov et al., 1996; Rudner and Losick, 2002). The A protein anchors the processing complex in this membrane and serves as a platform bringing B and BofA together, whereby, it has been proposed, BofA inhibits B (Rudner and Losick, 2002; Zhou and Kroos, 2004).

The proteolytic activation of pro-σ^K in the mother cell is controlled by a signal transduction pathway that emanates from the forespore after engulfment is complete (Rudner and Losick, 2001). The SpoIVB signaling protein (referred to as IVB), which is produced under the control of the forespore-specific transcription factor σ^G, is secreted into the space between the forespore and mother-cell membranes, where it overcomes the inhibition imposed on the B metalloprotease by A and BofA. (Figure 1A) (Cutting et al., 1991a). IVB is a serine protease, and a catalytically inactive mutant blocks σ^K activation in vivo (Hoa et al., 2002). It has been proposed that IVB triggers pro-σ^K processing by cleaving one (or more) of the proteins in the membrane protease complex. In support of this idea, during sporulation the degradation of A is IVB dependent, and IVB is capable of cleaving A in an in vitro transcription-translation reaction mixture (Dong and Cutting, 2003; Kroos et al., 2002; Zhou and Kroos, 2005).

To further investigate how IVB regulates intramembrane proteolysis, we have reconstituted IVB-dependent cleavage of A in vitro with purified components. We demonstrate that IVB cleaves the extracellular domain of A at four sites in vitro and that two of these cleavages are important to trigger pro-σ^K processing in vivo. Our data indicate that IVB-dependent cleavage at either one of these two sites is sufficient to activate the membrane-embedded protease. In addition, our biochemical analysis supports a model in which IVB-dependent cleavage of A triggers intramembrane proteolysis by a conformational change in the membrane protease complex. We present evidence that when IVB is unable to cleave A it can still activate pro-σ^K processing through a second serine protease, CtpB. Finally, we show that CtpB, like IVB, triggers σ^K activation by cleaving A. We propose that the IVB serine protease activates intramembrane proteolysis directly by cleaving the extracellular domain of A and indirectly by activating a second serine protease that, in turn, targets the same regulatory protein. This second pathway is reminiscent of the serine protease cascades found in blood clotting and dorsal-ventral patterning in *Drosophila* (Davie et al., 1991; Hecht and Anderson, 1992). A striking feature of this signaling pathway is the redundancy both within and between the two downstream branches.

Results

The IVB Signaling Protease Cleaves A at Multiple Sites In Vitro

Expression of *IVB* in *E. coli* is toxic and results in low levels of the recombinant protein (Wakeley et al., 2000). For this reason, all previous biochemical studies of the signaling protease have been performed using IVB protein derived from in vitro transcription-translation reactions. The experiments that follow were made possible by a new protocol for expressing and purifying highly active IVB signaling protease from *E. coli* (see Supplemental Data and Figure S1, both available with this article online).

It has been reported that IVB can cleave the A protein in vitro using proteins derived from in vitro transcription-translation reactions (Dong and Cutting, 2003). To assess whether IVB could cleave the A protein in an in vitro assay using purified components, we used the extracellular domain of A as a substrate. The A protein contains a single transmembrane segment with a large (~20 kDa)

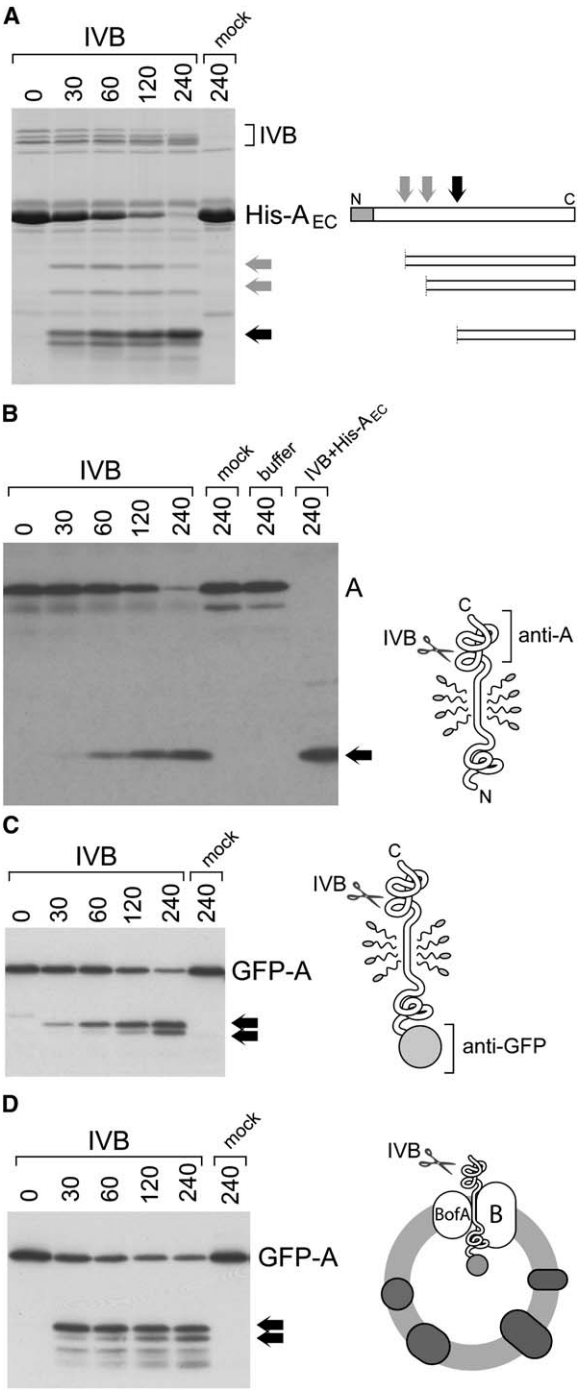

Figure 2. The IVB Signaling Protease Cleaves A at Multiple Sites

(A) IVB cleaves the extracellular domain of A (A_{EC}) in vitro. Purified His_6-A_{EC} was incubated with IVB (or the mock purification). Reactions were analyzed by SDS-PAGE and stained with Coomassie blue. Arrows indicate major (black) and minor (gray) C-terminal cleavage products. The autoproteolysis of IVB is visible at the top of the gel (see Figure S1C). A schematic representation of the extracellular domain of A (white rectangle), the His_6 tag (gray rectangle), cleavage sites, and C-terminal cleavage products is shown.

(B) IVB cleaves the full-length A protein. Detergent-solubilized membrane proteins from *B. subtilis* sporulating cells (BNC689) were incubated with IVB, mock, or buffer. Reactions were analyzed by immunoblot using anti-A antibodies. A cleavage reaction with the purified extracellular domain of A is shown for comparison. A schematic representation of the full-length A protein solubilized with detergent is shown. The C-terminal portion of the protein recognized by the anti-A antibodies is indicated. A scissor represents the approximate location of the IVB-dependent cleavage.

(C) IVB cleaves full-length GFP-A. Detergent-solubilized membrane proteins from *B. subtilis* sporulating cells containing a GFP-A fusion (BNC694) were incubated with IVB. Substrate and N-terminal cleavage products (arrows) were visualized by immunoblot using anti-GFP antibodies.

(D) IVB cleaves the full-length A protein in the context of the pro-σ^K signaling complex. Crude membrane vesicles prepared from strain BNC694 were incubated with IVB, and the reactions were analyzed by immunoblot using anti-GFP antibodies. IVB-dependent cleavage products are indicated (arrows). A schematic diagram of a membrane vesicle containing the signaling complex (GFP-A, B, and BofA) and other membrane proteins (dark gray ovals) is shown. All reactions were performed at room temperature for indicated times (in minutes).

extracellular domain at its C terminus (Green and Cutting, 2000). This domain is predicted to reside in the space between the mother-cell and forespore membranes (Rudner and Losick, 2002) and therefore should be accessible to the IVB signaling protease in vivo. An N-terminal hexahistidine tag was fused to the extracellular domain (referred to as $His\text{-}A_{EC}$), and this soluble fusion was purified by affinity chromatography on Ni^{2+}-agarose. Purified $His\text{-}A_{EC}$ was then incubated with the IVB protease, and the reaction products were resolved by SDS-PAGE. In confirmation of the previously reported experiments, IVB efficiently cleaved $His\text{-}A_{EC}$. We could detect one major and two minor cleavages (Figure 2A), resulting in three C-terminal cleavage products (see below). Importantly, the major C-terminal cleavage product was approximately the same size (~13 kDa) as the cleavage product observed in the in vitro transcription-translation reactions (Dong and Cutting, 2003). Finally, no cleavage products were detected when $His\text{-}A_{EC}$ was incubated with mock-purified protein (see Supplemental Data) or with buffer alone (Figure 2A and data not shown).

To investigate whether IVB could cleave the full-length membrane protein and to develop an in vitro assay to study IVB-dependent cleavage of A, we used detergent-solubilized membrane fractions from sporulating *B. subtilis* as a source of the A protein. To do this, crude membrane vesicles were isolated from a IVB mutant (strain BNC689) on a sucrose step gradient, and the membrane proteins were then solubilized with the nonionic detergent digitonin (see Experimental Procedures). We have previously shown that the A protein is efficiently solubilized under these conditions (Rudner and Losick, 2002). The purified IVB protease was incubated with the detergent-solubilized membrane proteins, and the cleavage reaction was analyzed by immunoblot using anti-A antibodies. In support of the idea that IVB cleaves the full-length A protein, a single cleavage product accumulated during the reaction time course (Figure 2B). Because the anti-A antibodies were raised against a portion of the extracellular domain of A (Resnekov et al., 1996), only the C-terminal cleavage product could be detected. Importantly, this product was indistinguishable in size from the major C-terminal cleavage product observed using $His\text{-}A_{EC}$ as a substrate (Figure 2B). To analyze the N-terminal cleavage

products in this assay, we used a strain (BNC694) harboring a functional N-terminal GFP fusion to the A protein (GFP-A) (Rudner and Losick, 2002). Using anti-GFP antibodies, we detected two N-terminal cleavage products (Figure 2C). The larger species appeared with similar kinetics to the C-terminal cleavage product (compare Figures 2B and 2C) and was derived from the same cleavage (see below). The smaller product appeared at later time points and was derived from cleavage of the larger one. For reasons that remain unclear, detection of the C-terminal cleavage product using the anti-A antibodies was variable. Consequently, in the remaining experiments we monitored the N-terminal reaction products using the GFP-A fusion and anti-GFP antibodies.

Finally, to investigate whether IVB cleaves GFP-A in the context of the signaling complex in membranes, we incubated purified IVB with crude membrane vesicles isolated from strain BNC694. The GFP-A protein in these membrane vesicles resides in a complex with B and BofA because all three proteins could be efficiently coimmunoprecipitated after detergent solubilization (Figure S2). We detected two cleavage products in this assay (Figure 2D). Importantly, these products were indistinguishable in size from the ones generated in the detergent-solubilized membrane assay described above. In addition to these two products, several smaller species accumulated over time. These species are probably due to nonspecific proteases present in the membrane vesicles because they were also detected (with longer exposures) in the reactions containing mock-purified protein (data not shown). In summary, we have shown that the IVB signaling protease can cleave the extracellular domain of A at multiple sites in vitro using purified components and when it is in a complex with B and BofA in membrane vesicles.

Identification of the IVB-Dependent Cleavage Sites in A

To determine the IVB-dependent cleavage sites in A, a preparative cleavage reaction was performed with His-A_{EC}, and the three cleavage products were subjected to N-terminal peptide sequencing. In addition, the same reaction was separated by reverse phase HPLC, and the eluted products were monitored by mass spectrometry (MS) (see Experimental Procedures). Four cleavage sites were identified in the extracellular domain of A (Figure 3A). The most abundant species detected by MS were generated by cleavages at two positions: position 1 and position 2, which we refer to as the major cleavage sites. The major C-terminal cleavage product in our assay with His-A_{EC} (Figure 2A) was generated by cleavage at position 1. Apparently, cleavage at position 2 follows this cleavage, because we could not detect a C-terminal product that was only cleaved at the second major cleavage site. However, the product resulting from cleavages at both major sites was an abundant species. The two minor cleavage products observed in the in vitro cleavage assay with His-A_{EC} were generated by cleavages at the two minor sites, positions 3 and 4. Comparison of all the known cleavage sites recognized by IVB indicates that, other than a small hydrophobic residue in the P1 position, there is no obvious recognition sequence for this protease (Figure 3A). The only exception is the second cleavage site in A and the cleavage site in the sporulation protein SpoIIQ (IIQ) (Jiang et al., 2005) (K. Marquis, N.C., T. Doan, and D.Z.R., unpublished data), which share significant similarity (Figure 3A).

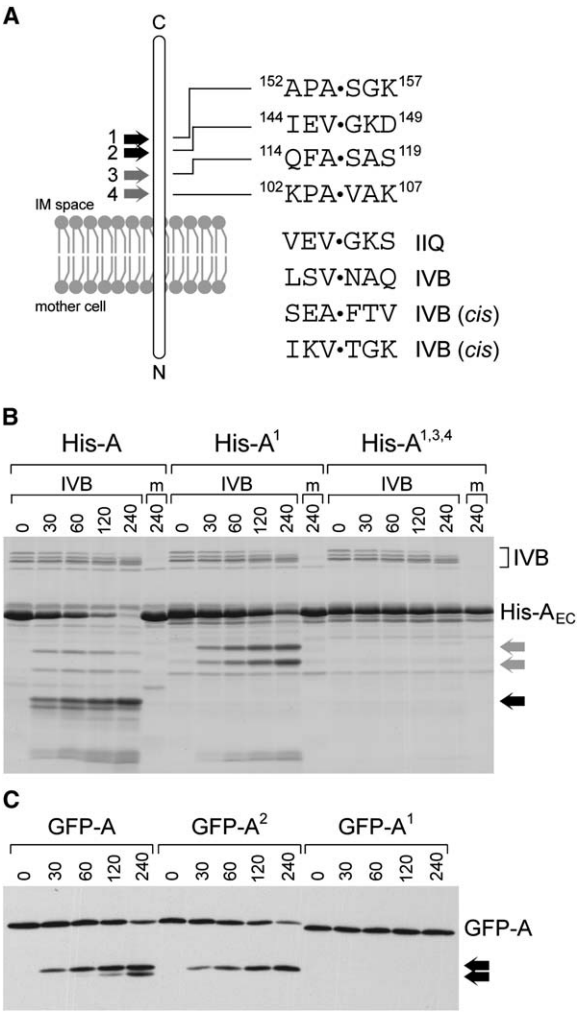

Figure 3. IVB-Dependent Cleavage Sites in A

(A) Schematic diagram of the full-length A protein and the IVB-dependent cleavage sites identified by N-terminal peptide sequencing and MS. The amino acids flanking the cleavage sites and their positions in the full length A protein are shown. Arrows indicate the position of two major (1 and 2, black) and two minor (3 and 4, gray) cleavage sites. For comparison, the IVB-dependent cleavage site of IIQ and the IVB autoproteolytic cleavage sites (Wakeley et al., 2000) are shown.

(B) Purified His-A and cleavage site 1 mutant (S155R), His-A^1; and cleavage sites 1, 3, and 4 mutant (S155R, S117R, and A106R), His-$A^{1,3,4}$, were incubated with IVB or the mock purification (m) for indicated times (in minutes). Reactions were analyzed by SDS-PAGE and stained with Coomassie blue. Arrows indicate one major (black) and two minor (gray) cleavage products. The anomalous mobility of the minor cleavage products in the His-A^1 cleavage reaction resulted from the S155R substitution.

(C) Detergent-solubilized membrane proteins from strains BNC694 (GFP-A), BNC861 (GFP-A^2), and BNC887 (GFP-A^1) were incubated with IVB for the indicated times (in minutes) and analyzed by immunoblot with anti-GFP antibodies. The cleavage products are indicated by arrows. The change in mobility of GFP-A^1 was due to the S155R substitution.

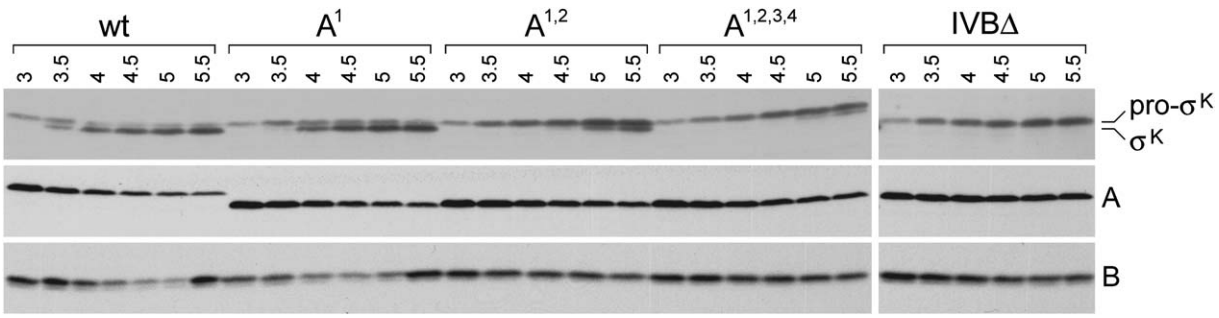

Figure 4. Uncleavable A Mutants Impair Pro-σ^K Processing In Vivo

Wild-type (BNC243), a IVB mutant (BNC692), and strains harboring the indicated uncleavable A mutants (BNC681, BNC848, and BNC850) were induced to sporulate by resuspension. At indicated times after the initiation of sporulation (in hours), samples were collected. Whole-cell extracts were prepared and analyzed by immunoblot using anti-σ^K, anti-A, and anti-B antibodies. The change in mobility of the A mutants was due to the S155R substitution.

Uncleavable A Mutants Impair Pro-σ^K Processing In Vivo

To investigate whether IVB-dependent cleavage of A is important to trigger pro-σ^K processing, we needed to generate uncleavable A mutants and assess whether these mutants impaired σ^K activation during sporulation. To this end, amino acid substitutions were introduced in the residues flanking the major and minor cleavage sites. These mutants were made in the context of the His-A_{EC} fusion and used as substrates in our in vitro cleavage assay. Interestingly, the mutant at cleavage position 1 (S155R) abolished cleavage at both major sites (His-A^1 in Figure 3B). This result is consistent with the idea that cleavage at position 2 depends on cleavage at position 1. In addition, the abundance of the two minor cleavage products increased in this mutant (Figure 3B). Amino acid substitutions in cleavage position 2 (V146F, G147Q), which prevented cleavage at this site (see below), had no effect on cleavage at position 1 and only modestly changed the profile of cleavage products (data not shown). Finally, amino acid substitutions in cleavage positions 1, 3, and 4 blocked all detectable cleavage by the IVB protease (His-$A^{1,3,4}$ in Figure 3B).

To test whether these mutants blocked cleavage by IVB in the context of the full-length A protein, the same mutations were introduced into the *gfp-A* fusion. Solubilized membranes derived from sporulating cells (strains BNC694, BNC861, and BNC887) were incubated with purified IVB and analyzed by immunoblot. The cleavage site 2 mutant blocked the appearance of the smaller cleavage product, while a cleavage position 1 mutant (or positions 1 and 2 together) prevented all detectable cleavage by IVB (Figure 3C and data not shown).

With uncleavable A mutants in hand, we could now address whether IVB-dependent cleavage of A was important to trigger pro-σ^K processing in vivo. To do this, the mutations were made in the context of the untagged *A* gene and introduced into *B. subtilis*. These strains were induced to sporulate, and samples were collected every half hour for immunoblot analysis. In wild-type cells (BNC243), 50% of pro-σ^K was processed by hour 3.5 of sporulation, and greater than 80% was converted to mature σ^K 30 min later (Figure 4). Moreover, the level of A and B decreased around the same time as processing commenced. In a mutant blocked for cleavage at position 1 (strain BNC681), accumulation of σ^K was reproducibly delayed by 30 min (A^1 in Figure 4). Virtually no mature σ^K was detected at hour 3.5, and 50% was processed by hour 4. In addition, the A protein was slightly more stable in this mutant background (Figure 4). These results indicate that IVB-dependent cleavage at position 1 is important for efficient processing of pro-σ^K. However, in the absence of the IVB protease (strain BNC692), pro-σ^K processing was completely blocked and the A protein was stabilized (Figure 4) (Dong and Cutting, 2003; Kroos et al., 2002). These observations suggest that IVB can cleave A at other sites when position 1 is blocked. To determine whether IVB also uses the minor cleavage sites to trigger σ^K activation, we analyzed pro-σ^K processing in the triple mutant blocked for cleavage at positions 1, 3, and 4. In vitro, this mutant was completely immune to the IVB protease. However, in vivo, pro-σ^K processing was still delayed by only 30 min (Figure S3). Although, in our in vitro assays, blocking IVB-dependent cleavage at position 1 also blocked cleavage at position 2 (Figures 3B and 3C), we tested the double mutant (strain BNC848). Strikingly, in this background, 50% of pro-σ^K was converted to σ^K at hour 5.5 ($A^{1,2}$ in Figure 4), a delay of ~2 hr. Apparently, in vivo these two sites can be cleaved independently by IVB. Finally, when we combined all four cleavage site mutants (strain BNC850), pro-σ^K processing was even slightly more delayed ($A^{1,2,3,4}$ in Figure 4). Moreover, in these last two mutants, the level of A (and B) remained high, supporting the idea that IVB cleaves A at these sites in vivo (Figure 4). Importantly, all of the A mutants that impaired the timing of pro-σ^K processing could be bypassed by a mutation in *bofA* (data not shown). This result indicates that these uncleavable mutants do not possess new (neomorphic) inhibitory function. Taken together, our results indicate that IVB can cleave A at multiple sites in vivo and these cleavages are important to relieve the inhibition imposed on the B processing enzyme.

IVB-Dependent Cleavage of A Does Not Disrupt the Signaling Complex

How does IVB-dependent cleavage of A relieve the inhibition imposed on the processing enzyme? Since A is

required to assemble the signaling complex, an appealing model is that cleavage of A releases B or BofA (or both) from the complex, which results in activation of the processing enzyme (Dong and Cutting, 2003; Kroos et al., 2002; Zhou and Kroos, 2004). To investigate this model biochemically, we took advantage of our membrane-cleavage assays. Figure 2D shows that after IVB cleaved GFP-A in crude membrane vesicles, the membrane-anchored GFP-A cleavage products were further proteolyzed by other (presumably nonspecific) proteases present in the vesicles. Importantly, GFP-A was only weakly proteolyzed after the 4 hr incubation with mock-purified protein (Figure 2D). Thus, these nonspecific proteases cleave GFP-A much more efficiently after it has been cleaved by IVB. To assess whether GFP-A remains complexed with BofA after IVB-dependent cleavage, we used these nonspecific proteases as a probe. We reasoned that if BofA remains in a complex with GFP-A after IVB-dependent cleavage, then it might protect the GFP-A cleavage products from further proteolysis. To test this, IVB was incubated with membrane vesicles derived from the GFP-A fusion strain (BNC694) and the same strain lacking BofA (BNC889). In both reactions, full-length GFP-A was cleaved by IVB with very similar kinetics as judged by loss of the full-length substrate (Figure 5A). However, the membrane-associated cleavage products were more rapidly and extensively degraded in the absence of BofA (Figure 5A). The degradation of GFP-A was not simply due to the absence of BofA, since there was no significant degradation of GFP-A in the mock-treated reaction (Figure 5A). Thus, BofA protects the membrane-associated GFP-A cleavage product from further proteolysis. These results suggest that BofA remains complexed with the membrane-anchored portion of A after IVB cleaves its extracellular domain. Since IVB cleavage likely removes most of the extracellular domain of A, these results further suggest that A and BofA interact, in part, through their transmembrane domains.

To investigate whether the association between B and A is perturbed after IVB-dependent cleavage, we monitored the stability of B in membrane vesicles. We reasoned that if B is released from the complex or changes conformation after IVB-dependent cleavage of A, it might also be susceptible to the nonspecific proteases present in the membrane vesicles. For these experiments, we used a strain (BNC635) harboring a functional GFP fusion to B (B-GFP) (Rudner and Losick, 2002), which was easier to detect by immunoblot (using anti-GFP antibodies) than the untagged protein. During IVB-dependent cleavage of A, we monitored the level of B-GFP in the membrane vesicles. Over the reaction time course, the level of B-GFP decreased (Figure 5B). Importantly, B-GFP remained stable in the presence of an uncleavable A mutant (strain BNC639), even after 4 hr of incubation with IVB (Figure 5B).

These protease susceptibility experiments indicate that IVB-dependent cleavage of A either causes dissociation of B from A or causes a conformational change in the signaling complex. To distinguish between these two models, we investigated whether A and B remain complexed after IVB cleaves A. To do this, we took advantage of the fact that A and B stably interact and can be efficiently coimmunoprecipitated as a complex

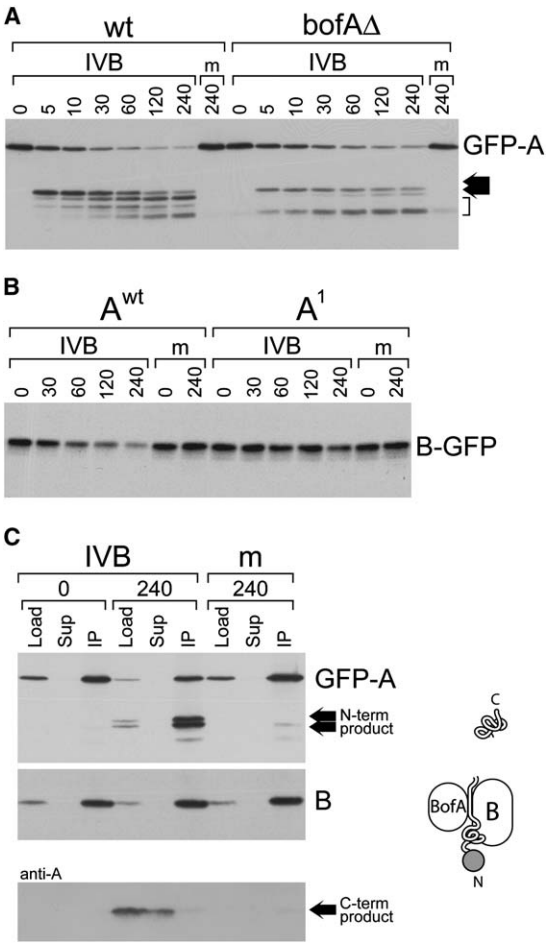

Figure 5. IVB-Dependent Cleavage of A Does Not Disrupt the Signaling Complex

(A) BofA protects GFP-A from further proteolysis after IVB-dependent cleavage. Crude membrane vesicles derived from wild-type (BNC694) and *bofA*Δ (BNC889) strains were incubated with IVB or the mock purification (m) for indicated times (in minutes). Full-length GFP-A, N-terminal cleavage products (arrows), and further proteolyzed species (bracket) were monitored by immunoblot using anti-GFP antibodies.

(B) B-GFP becomes susceptible to proteolysis after IVB-dependent cleavage of A. Crude membrane vesicles were prepared from wild-type (A^{wt}; BNC635) and an uncleavable A mutant (A^1; BNC639). Both strains harbor a functional *B-GFP* fusion. Membrane vesicles were incubated with IVB or the mock purification (m) for indicated times (in minutes). The stability of B-GFP was monitored by immunoblot using anti-GFP antibodies.

(C) B and A remain complexed after IVB-dependent cleavage. Immunoblots from coimmunoprecipitation assays after IVB-dependent cleavage of A. Detergent-solubilized membrane proteins from a strain (BNC694) harboring a *GFP-A* fusion were incubated with IVB or the mock purification (m). At indicated times (in minutes), the interaction between GFP-A and B was analyzed by coimmunoprecipitation using anti-GFP antibody resin. The cleavage reactions prior to immunoprecipitation (Load), the supernatants after immunoprecipitation (Sup), and the immunoprecipitations (IP) were subjected to immunoblot analysis using anti-GFP, anti-B, and anti-A antibodies. The N- and C-terminal cleavage products are indicated by arrows. Equivalent amounts of the load and supernatant (and approximately five times more of the IP) were analyzed. A schematic representation of the signaling complex after IVB-dependent cleavage of A is shown.

after detergent solubilization (Rudner and Losick, 2002). Importantly, after detergent solubilization, B is no longer susceptible to the nonspecific proteases in the membrane vesicles. First, detergent-solubilized membrane fractions derived from a GFP-A strain (BNC694) were incubated with the IVB protease. After 4 hr, when greater than 75% of GFP-A was cleaved (Figure 5C), the GFP-tagged fusion was immunoprecipitated using anti-GFP resin. The immunoprecipitate was then analyzed for the presence of B by immunoblot. Strikingly, even when 75% of GFP-A was cleaved, virtually all of the B processing enzyme was coimmunoprecipitated (Figure 5C). This result indicates that the membrane-anchored GFP-A cleavage product still efficiently interacts with B. By probing these immunoprecipitates with anti-A antibodies, we also discovered that the extracellular domain of A was released (and found in the supernatant) after cleavage by IVB (Figure 5C). All together, our results indicate that IVB-dependent cleavage of A releases the extracellular domain of A but does not disrupt the complex composed of A, B, and BofA (Figure 5C). Thus, this analysis suggests that relief of inhibition does not involve dissociation of the processing enzyme B from its negative regulators. Rather, our data are consistent with a model in which IVB-dependent cleavage of A causes a conformational change in the signaling complex that triggers pro-σ^K processing activity.

A Second Serine Protease Is Required to Trigger Pro-σ^K Processing and Is a Target of IVB Action

We have shown that a quadruple mutant in A that cannot be cleaved by IVB delays pro-σ^K processing by ~2 hr (Figure 4). Since a IVB mutant completely blocks σ^K maturation, this result indicates that a second pathway under the control of IVB can activate pro-σ^K processing. We wondered whether this second pathway might involve a second signaling protease, CtpB. CtpB is a secreted serine protease with a domain structure that resembles IVB (Pan et al., 2003). A CtpB mutant delays pro-σ^K processing by ~30 min (Figure 6A) (Pan et al., 2003), and recently CtpB has been implicated in the degradation of BofA (Zhou and Kroos, 2005). To determine whether the residual pro-σ^K processing observed in the $A^{1,2,3,4}$ mutant was due to the action of CtpB, we monitored σ^K maturation in the quadruple mutant lacking CtpB. Strikingly, mature σ^K was undetectable in this strain even after 7 hr of sporulation (Figure 6A). Thus, when the extracellular domain of A cannot be cleaved by IVB, CtpB becomes essential for pro-σ^K processing. Furthermore, this block in pro-σ^K processing resulted in a 50-fold reduction in sporulation efficiency (Figure 6A). This sporulation defect is 26-fold lower than the multiplicative effect of either mutant on its own (Figure 6A). Importantly, similar synthetic phenotypes were observed using the A mutant blocked at only cleavage positions 1 and 2 or a catalytic mutant of CtpB ($CtpB^{S309A}$) (data not shown). Taken together, we conclude that efficient pro-σ^K processing requires both IVB-dependent cleavage of A and the protease activity of CtpB.

These results favor a model in which IVB stands at the top of two pathways that can control pro-σ^K processing. In this model, IVB has two targets: A and CtpB. The IVB-dependent cleavage of CtpB would activate this second serine protease in a manner analogous to the serine protease cascade in blood clotting (Davie et al., 1991). Thus, in the uncleavable A mutant, IVB could still activate CtpB, leading to the activation of pro-σ^K processing. To investigate this model, we raised antibodies against CtpB and monitored its state during sporulation. In wild-type cells, full-length CtpB was converted to a smaller form shortly after its synthesis (Figure 6B). In support of the idea that CtpB is indeed a target of IVB, this cleavage was IVB dependent (Figure 6B). Moreover, the cleavage of CtpB was coincident with IVB accumulation and preceded pro-σ^K processing (Figure 6B). We note that in the absence of IVB, a small amount of CtpB was converted to an intermediate-sized product (Figure 6B). However, this is probably due to autoproteolysis, as we did not detect this cleavage product in the catalytic mutant $CtpB^{S309A}$ (Figure S4). All together, our data support a model in which IVB controls σ^K activation through two pathways. In this model, IVB triggers pro-σ^K processing directly by cleaving the extracellular domain of A and indirectly by activating the second serine protease, CtpB (Figure 1B).

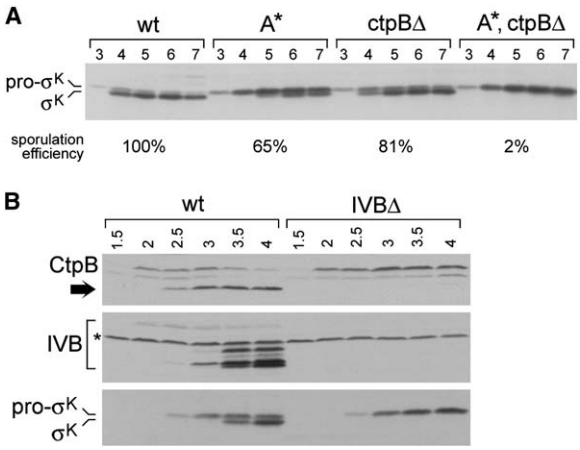

Figure 6. CtpB Is Required to Trigger Pro-σ^K Processing and Is a Target of IVB Action

(A) CtpB is necessary for pro-σ^K processing when IVB cannot cleave A. Wild-type (BNC243), the A mutant blocked at all four cleavage positions (A*; BNC850), a *ctpB* mutant (ctpBΔ; BNC734), and the double mutant (A*, ctpBΔ; BNC867) were induced to sporulate by resuspension. At indicated times (in hours) after the initiation of sporulation, samples were collected. Whole-cell extracts were analyzed by immunoblot using anti-σ^K antibodies. The sporulation efficiency of each strain (relative to wild-type) is shown.

(B) CtpB is cleaved during sporulation in a IVB-dependent manner. CtpB, IVB, and pro-σ^K processing during sporulation in wild-type (BNC949) and a *IVB* mutant (IVBΔ; BNC953) were analyzed by immunoblot. The CtpB cleavage product (arrow), the autoproteolyzed IVB products (bracket), and a nonspecific band (asterisk) that cross-reacts with the anti-IVB antibodies are indicated.

CtpB Triggers Pro-σ^K Processing by Cleaving A

The results of Figure 6A indicate that CtpB can activate pro-σ^K processing when IVB is unable to cleave A. To determine how CtpB triggers processing, we purified a CtpB-His_6 fusion synthesized in *E. coli* (see Experimental Procedures). Recently, Zhou and Kroos have proposed that CtpB acts by cleaving and/or degrading

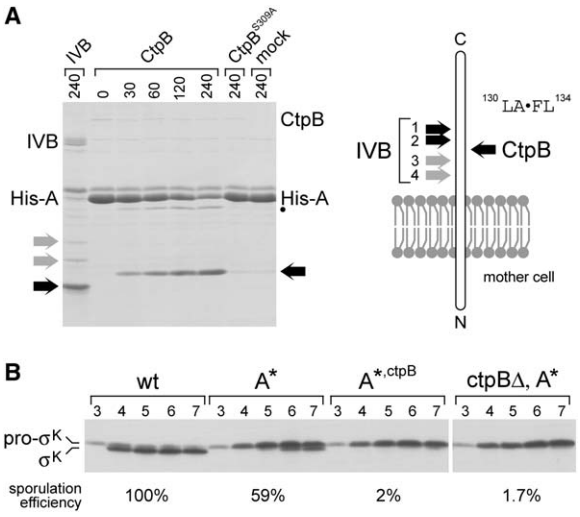

Figure 7. CtpB Activates Pro-σ^K Processing by Cleaving A

(A) CtpB cleaves the extracellular domain of A in vitro. His_6-A_{EC} was incubated with purified CtpB, $CtpB^{S309A}$, IVB, or mock-purified protein (mock) at room temperature for indicated times (in minutes). Reactions were analyzed by SDS-PAGE and stained with Coomassie blue. The arrows indicate the major (black) and minor (gray) C-terminal cleavage products. A dot (●) indicates the cleavage product resulting from CtpB-dependent cleavage in a region of His-A_{EC} derived from vector sequence between the His_6 tag and the extracellular domain of A. A schematic representation of the CtpB-dependent and IVB-dependent cleavage sites in the context of the full-length A protein is shown. The amino acids flanking the CtpB-dependent cleavage site in A, as determined by MS, are indicated.

(B) Blocking CtpB-dependent and IVB-dependent cleavage of A prevents pro-σ^K processing in vivo. Wild-type (BNC243), the A mutant blocked at all four IVB-dependent cleavage sites (A*; BNC850), the A mutant blocked at all four IVB-dependent cleavage sites *and* the CtpB-dependent cleavage site (A131R) ($A^{*,ctpB}$; BNC910), and a *ctpB* null strain harboring the A mutant blocked at all four IVB-dependent cleavage sites (ctpBΔ, A*; BNC867) were induced to sporulate by resuspension. At indicated times (in hours) after the initiation of sporulation, samples were collected. Whole-cell extracts were analyzed by immunoblot using anti-σ^K antibodies. The sporulation efficiency of each strain (relative to wild-type) is shown. The immunoblot was from the same gel (and the same autoradiography film), but control lanes were removed for clarity.

BofA (Zhou and Kroos, 2005). However, we could not detect CtpB-dependent cleavage of BofA in our in vitro assay (data not shown). To investigate whether CtpB targets the extracellular domain of A, we incubated the purified protease with His-A_{EC}. Two cleavage products were generated during the reaction time course (Figure 7A). Analysis of the cleavage reaction by MS revealed that the larger product resulted from cleavage of the fusion protein in a region derived from vector sequence between the hexahistidine tag and the extracellular domain of A (data not shown), while the smaller product resulted from cleavage after amino acid 131 between the IVB protease cleavage positions 2 and 3 (Figure 7A). This cleavage product was not detected when His-A_{EC} was incubated with mock-purified protein or with a CtpB catalytic mutant ($CtpB^{S309A}$) (Figure 7A). Importantly, CtpB was also capable of cleaving the His-A_{EC} mutant (His-$A^{1,3,4}$) that could not be cleaved by the IVB protease (data not shown). All together, our results indicate that, in vitro, both signaling serine proteases target the extracellular domain of A.

To determine whether CtpB-dependent cleavage of A triggers pro-σ^K processing, we substituted alanine 131 with arginine in the A quadruple mutant. This substitution blocked CtpB-dependent cleavage at this site in vitro (data not shown). Accordingly, this quintuple mutant should be immune to cleavage by both IVB and CtpB in vivo. A strain (BNC910) harboring the quintuple mutant was induced to sporulate, and pro-σ^K processing was monitored by immunoblot. Remarkably, mature σ^K remained undetectable for the full 7 hr time course (Figure 7B). Moreover, the sporulation efficiency of this mutant was reduced 50-fold compared to wild-type (Figure 7B). Thus, this uncleavable mutant phenocopied the *ctpB* null strain harboring the A quadruple mutant (BNC850) (Figure 7B). These results demonstrate that when IVB is unable to cleave A, CtpB triggers intramembrane proteolysis by cleaving the very same regulatory protein.

Discussion

We have presented evidence that the IVB signaling protease sits at the apex of two pathways that govern the activation of the mother-cell transcription factor σ^K (Figure 1B). In the first and principal pathway, we have shown that IVB triggers pro-σ^K processing directly by cleaving the extracellular domain of A at either of two partially redundant sites. Our data suggest that this cleavage causes a conformational change in the signaling complex that activates RIP. In the second pathway, we propose that the IVB serine protease triggers pro-σ^K processing indirectly by cleaving and activating a second serine protease, CtpB. CtpB, in turn, targets the extracellular domain of A. Thus, the two protease pathways converge on the same regulatory protein. The second pathway is reminiscent of the serine protease cascades found in blood clotting and dorsal-ventral patterning in *Drosophila* (Davie et al., 1991; Hecht and Anderson, 1992). In these pathways, a serine protease is secreted as an inactive zymogen and is subsequently activated by an upstream serine protease.

Fail-Safe Mechanisms Ensure the Timely Activation of σ^K

B. subtilis cells have evolved multiple levels of regulation to prevent the inappropriate activation of σ^K. Activation of this transcription factor involves a mother-cell-specific genomic rearrangement within the *pro-σ^K* gene (Stragier et al., 1989), temporal and spatial transcriptional regulation (Kunkel et al., 1989), and finally, posttranslational regulation at the level of precursor processing (Cutting et al., 1990). Indeed, natural selection has gone through great lengths to ensure that σ^K is not activated at the wrong time or place. Here, we show that once the time is right, the sporulating cell also takes no chances. First, we have shown that the forespore signaling protein IVB cleaves A at two sites, either of which is sufficient to trigger pro-σ^K processing. And second, we presented evidence that IVB can activate pro-σ^K processing through two separate pathways that converge on the regulatory protein A. Thus, not only is the premature activation of σ^K tightly controlled, but also fail-safe

mechanisms exist to ensure its timely activation. A fail-safe mechanism involving redundant cleavage sites is also employed during the cell cycle in the budding yeast *S. cerevisiae*. A key step in the metaphase to anaphase transition is the regulated degradation of the cohesin complex that holds sister chromatids together (Uhlmann et al., 1999). The ESP1 separin protease (also called separase) cleaves the cohesin subunit Scc1 at two sites, either of which is sufficient to trigger its further degradation (Uhlmann et al., 2000). Degradation of Scc1 causes release of the sister chromatids, driving the cells into anaphase. As in the case of IVB-dependent cleavage of A, redundant cleavage sites in Scc1 ensure timely passage through the cell cycle.

How Does IVB-Dependent Cleavage of A Trigger Processing Activity?

Our in vitro biochemical analysis supports a model in which IVB-dependent cleavage of A causes a conformational change in the signaling complex, thereby triggering pro-σ^K processing. However, our data cannot rule out a model in which IVB-dependent cleavage of A makes A susceptible to degradation by other proteases, resulting in the release of B or BofA. This "degradation-as-trigger" model has been proposed by two groups and is based on the observation that A is degraded in a IVB-dependent manner (Figure 4) (Dong and Cutting, 2003; Kroos et al., 2002; Zhou and Kroos, 2005). However, in these studies the level of the B processing enzyme was not examined. Instead, a stable B-GFP fusion was analyzed using anti-GFP antibodies (Kroos et al., 2002). Here, we show that during sporulation, both A and B are degraded in a IVB-dependent manner (Figure 4). Moreover, their degradation follows similar kinetics suggesting that the disappearance of B occurs at the same time as A. Since processing of pro-σ^K by B must occur prior to its degradation, the degradation-as-trigger model seems to us unlikely. Instead, we favor a model in which the degradation of A (and B) is a consequence of signaling (a result of the conformational change in the complex) rather than the activating event. Reconstitution of pro-σ^K processing in vitro and IVB-dependent relief of its inhibition by A and BofA will be required to ultimately distinguish between these two models.

CtpB Triggers Pro-σ^K Processing by Targeting A

We have shown that when IVB cannot cleave A, CtpB can activate pro-σ^K processing by cleaving it instead. In wild-type sporulating cells, where both IVB and CtpB can cleave A, both proteases could act on the same substrate protein, or they could target different signaling complexes. In the first scenario, further cleavage of the membrane-anchored fragment of A by CtpB could stimulate activation of the processing enzyme B. In the second scenario, IVB-dependent activation of CtpB could serve to amplify the forespore signal. In support of this second model, CtpB was capable of cleaving the A quadruple mutant that could not be cleaved by IVB (data not shown). In addition, we have found that the wild-type A protein is stabilized in sporulating cells lacking CtpB (Figure S5). These results suggest that CtpB can target intact A molecules.

Recently, Zhou and Kroos have reported that CtpB participates in activating pro-σ^K processing by promoting the degradation of the BofA inhibitor (Zhou and Kroos, 2005). Although we could not detect CtpB-dependent cleavage of BofA in vitro, it is possible that CtpB cleaves both A and BofA in vivo. Our branched pathway model is certainly compatible with CtpB cleaving both proteins, but our analysis of uncleavable A mutants suggests that the principal target of CtpB is A. The identification of CtpB-dependent cleavage sites in BofA and the generation of uncleavable mutants will be necessary to assess the contribution of CtpB-dependent cleavage of BofA in triggering pro-σ^K processing in vivo.

Finally, our in vivo data suggest that CtpB is activated by IVB-dependent cleavage. However, in the in vitro experiments described here, purified CtpB retained protease activity without prior cleavage by IVB (Figure 7A). There is precedent in the literature for uncleaved zymogens retaining protease activity in vitro (LeMosy et al., 2001). It is also possible that the CtpB activity could be due to the absence of its putative signal sequence, which we removed for synthesis in *E. coli*. Finally, in preliminary experiments we could detect some increase in CtpB protease activity after preincubation with IVB (N.C., H. Tukachinsky, and D.Z.R., unpublished data).

A Two-Step Proteolytic Cascade Analogous to Other Membrane-Embedded Proteases

The pro-σ^K processing enzyme B is a member of a family of membrane-embedded metalloproteases that includes S2P, required for the proteolytic activation of SREBP in mammals (Rawson et al., 1997), and RseP, involved in the activation of the stress-response sigma factor σ^E in *E. coli* (Alba et al., 2002; Kanehara et al., 2002). The activation of both of these I-CLiPs also requires a signaling protease on the opposite side of the membrane. These signaling proteases, which are unrelated serine proteases, are both required for substrate maturation: they cleave the extracellular (or luminal) domains of their substrates, which then become competent to be cleaved by their membrane-embedded protease partner. This first cleavage is referred to as ectodomain clearance. It is hypothesized that release of the extracellular domain allows the substrate to gain access to the caged interior of the I-CliP, where the catalytic site resides (Lemberg and Martoglio, 2004). In the case of the σ^K signaling pathway, pro-σ^K, which has a 20 amino acid prodomain, lacks an extracellular domain. Nonetheless, the processing of pro-σ^K is not constitutive, and in fact the regulation of the B processing enzyme shares similar features to that of S2P and RseP. Like these membrane metalloproteases, the activation of B requires a serine protease, IVB, on the opposite side of the membrane. Instead of cleaving the extracellular domain of pro-σ^K, the signaling protease cleaves the extracellular domain of A. Moreover, we have shown that IVB-dependent cleavage of A releases the extracellular domain from the signaling complex (Figure 5C). By analogy to the regulation of S2P and RseP, we speculate that ectodomain clearance of the A protein changes the conformation of the signaling complex such that pro-σ^K can gain access to the catalytic pocket of the B processing enzyme.

Experimental Procedures

General Methods

All *B. subtilis* strains were derived from the prototrophic strain PY79 and are listed in Table S1. Oligonucleotide primers used for PCR and site-directed mutagenesis are listed in Table S2. The plasmids used in this study are listed in Table S3. A description of their construction can be found in Supplemental Data. Sporulation was induced by resuspension or exhaustion (in supplemented DS medium). All fusion proteins were synthesized in *E. coli* BL21 DE3 pLysS and purified on Ni^{2+}-NTA agarose (Qiagen). Purification protocols can be found in Supplemental Data. The preparation of crude membrane vesicles from *B. subtilis* sporulating cells, the detergent solubilization of membrane proteins, and the coimmunoprecipitation of membrane protein complexes were performed as described previously (Rudner and Losick, 2002). Detailed protocols can be found in Supplemental Data. Immunoblots were performed as described previously (Rudner and Losick, 2002). Affinity-purified antibodies were diluted 1:10,000 (anti-IVB, anti-σ^K, anti-A, anti-GFP, and anti-CtpB) or 1:1000 (anti-B).

Determination of Cleavage Sites

To determine the N termini of the IVB-dependent His-A_{EC} cleavage products, the cleavage reactions were separated by SDS-PAGE and transferred to ProBlott PVDF (Applied Biosystems); the cleavage products were then subjected to Edman degradation by the Harvard Microchemistry Facility (Cambridge, Massachusetts). To determine the identity of the His-A_{EC} cleavage products, the IVB (or CtpB) cleavage reactions were loaded on a C18 column and eluted with a linear gradient of acetonitrile. The elution products were monitored by MS at the Taplin Biological Mass Spectrometry Facility at Harvard Medical School (Boston, Massachusetts).

Cleavage Assays

Approximately 1.5 μg of the purified His-A_{EC} substrates was mixed with ~100 ng of purified IVB (or 50 ng of CtpB) (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. Crude membrane vesicles (4 μl, equivalent to 125 μl of cells) or digitonin-solubilized membrane proteins (4 μl, equivalent to 250 μl of cells) were incubated with ~50 ng of IVB (per time point) at room temperature. The reactions were resolved by SDS-PAGE and analyzed by immunoblot.

Supplemental Data

Supplemental Data include five figures, three tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at www.molecule.org/cgi/content/full/23/1/25/DC1/.

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