The PDZ Domain of the SpoIVB Transmembrane Signaling Protein Enables cis-trans Interactions Involving Multiple Partners Leading to the Activation of the Pro-σ^K Processing Complex in Bacillus subtilis*§

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In sporulating cells of Bacillus subtilis, the serine peptidase SpoIVB is the essential component of a transmembrane signaling cascade between the two intracellular compartments (forespore and mother cell) that leads to activation of the σ^K transcription factor in the mother cell chamber. This regulatory process, referred to as the σ^K checkpoint, is essential for ensuring proper development of the spore and introduces an appropriate level of fidelity to the developmental process. This work unravels the signaling process and establishes how SpoIVB interacts with other protein partners in the σ^K checkpoint. SpoIVB is synthesized as a zymogen that is auto-proteolytically activated and carries a PDZ domain that is responsible for at least three distinct binding reactions, a phenomenon not previously demonstrated for an intramembrane PDZ domain. First, binding to the SpoIVB NH2 terminus to maintain the protein in its zymogen form. Second, following secretion across a spore membrane, binding in trans to the COOH terminus of another SpoIVB molecule. Binding in trans facilitates the first cleavage event of SpoIVB near the NH2 terminus releasing it from the inner forespore membrane. We show that at least two further cis cleavage events occur at specific sites near the NH2 terminus after which the PDZ domain targets SpoIVB to the pro-σ^K processing complex in the outer forespore membrane. Specifically, SpoIVB binds to the COOH terminus of BofA. In turn, this allows SpoIVB to cleave the COOH terminus of SpoIVFA an event pivotal to activating the SpoIVFB zinc metalloprotease by disruption of the heteroligomeric pro-σ^K complex.

Spoore formation in Bacillus subtilis is a highly coordinated process relying on differential gene expression being tightly coupled with morphogenesis. Minor errors in the activation of gene expression orchestrated by any one of the five sporulation-specific sigma factors (σ) can have catastrophic consequences on the completion of sporulation (1, 2). A notable example is the activation of the transcription factor, σ^K, in the mother cell chamber of the sporulating cell in what is referred to as the "σ^K checkpoint." The σ^K-regulon is involved in the final stages of spore maturation including spore coat biosynthesis and release of the spore from the sporangial cell. σ^K is made in an immature (proprotein) form known as pro-σ^K and is activated by proteolytic cleavage of its amino-terminal (pro) leader sequence (3, 4). Processing of pro-σ^K must occur at a precise time because premature activation of σ^K by as little as 30 min leads to aberrant spore formation (3). Pro-σ^K has been shown to interact with the outer forespore membrane (OFM) in which it is embedded a processing complex consisting of three peptidases, SpoIVFB, SpoIVFA, and BofA (5–7). Extensive cytological as well as biochemical analysis has recently shown that SpoIVFA, SpoIVFB, and BofA exist in a multimeric complex in the OFM (8). SpoIVFB has been identified as the zinc-metalloprotease that cleaves pro-σ^K and is anchored in the OFM by three transmembrane loops (9–12) and belongs to a new family of membrane-embedded metalloproteases whose active sites lie at the membrane interface (10, 11). Genetic experiments have shown that SpoIVFA and BofA negatively regulate the activity of SpoIVFB because mutations that disrupt the COOH termini of both SpoIVFA (bofB mutations) and BofA (bofa) allow constitutive processing of pro-σ^K (5, 6). Several models for how SpoIVFA and BofA might regulate SpoIVFB activity have been proposed but only recently has sufficient evidence validated a working model (8). These studies have shown first, that SpoIVFA plays a central role in assembling the pro-σ^K processing complex by serving as a platform for bringing BofA and SpoIVFB together as a heteroligomeric complex. Second, in this “tethered” state BofA inhibits the SpoIVFB processing enzyme, probably through a direct interaction, until the appropriate forespore signal is received that disrupts the processing complex. Finally, the COOH terminus of SpoIVFA that is exposed to the intramembrane space between the inner forespore membrane (IFM) and OFM is important for recruiting BofA because bofA mutations at the COOH-terminal tip of SpoIVFA prevent proper localization of BofA in the membrane.

Signaling of SpoIVFB-controlled processing, however, is mediated exclusively by SpoIVB, which is made in the opposed forespore chamber (13). Its gene, spoIVB, is initially expressed under the control of RNA polymerase associated with σ^K (Eo^K) but is subsequently transcribed in earnest under E0^F control (14). SpoIVB has been studied extensively and offers important insights into intercompartmental signaling. First, SpoIVB has been shown to be a serine peptidase of a new family of trypsin-like enzymes belonging to clan PA(S) with the MEROPS clas...
SpoIVB Transmembrane Signaling

4349

Sf9. Second, SpoIVB carries a PDZ domain (Fig. 1) that could be used for self-recognition, enabling cleavage in trans, as well as for interaction with its signaling partner(s) (18). SpoIVB must signal pro-o expression by acting across the IFM and this is achieved through its NH2-terminal hydrophobic signal sequence that is required for it to target and transit the IFM (15). Self-cleavage of SpoIVB is initially inhibited by the action of BofC (19, 20). This inhibition is brief and once the levels of SpoIVB exceed those of BofC then SpoIVB undergoes self-cleavage. At least one cleavage reaction of SpoIVB has been shown to be trans and is followed by further cleavage events generating a number of discrete SpoIVB species that are released from the IFM (15). Recently, we have shown that SpoIVB can cleave the COOH-terminal domain of SpoIVFA that is exposed in the space between the inner and outer forespore membranes (21). We have proposed that cleavage of this region of SpoIVFA would lead to disassembly of the pro-o sheet complex releasing BofA inhibition and activation of SpoIVB.

Here, we have examined autoproteolysis of SpoIVB in detail and determined the order and nature of self-cleavage reactions. We also show that the PDZ domain of SpoIVB is required for at least three separate interactions.

EXPERIMENTAL PROCEDURES

General Methods

General recombinant DNA work was as described in Sambrook and Russell (22) and Bacillus methods (sporulation, transformation, etc.) were as described in Harwood and Cutting (23).

Strains

The following B. subtilis congenic strains were used: PY79 (spo+ (24)), SC1836 (spoIVB::apc (25)), SC2080 (spovBSA378 chr::T9170HU144 (15)), and NH1135 (spovBSA::apc amye::spovBD149N (18)).

Expression Plasmids

pET28b (+) plasmids (Novagen) were used for expression of SpoIVB proteins. Vectors pJIVB, pJIVBSA378, and pJIVBD149N expressing M36, M36-S378A, and M36-D149N have been described previously (21) but in this work M36-S378A is referred to as M36* and M36-D149N as M36++. V100 that encodes the V100 SpoIVB polypeptide and is truncated for residues 1–99 has been reported elsewhere (21). To construct clones expressing full-length SpoIVB (M1) or truncated derivatives, N53, F63, and T75, two primers were used in each case to amplify the appropriate segment of spoIVB from PY79 (spo+) chromosomal DNA as outlined under Supplemental Materials Table SI. Primers carried restriction endonuclease sites to facilitate cloning in pET28b (+). To clone similar spoIVB templates but carrying the S378A or D149N mutations the same primers were used but with chromosomal DNA from strain SC2080 (spoIVBSA378) or NH1135 (spoIVBD149N) as the PCR template (Supplemental Materials Table SI). To construct vectors expressing COOH-terminal deletions of SpoIVB (M36Δ9, M36Δ16, M36Δ24, M36Δ43) a forward primer, 3F6, was used that was annealed to spoIVB starting at codon 36 (Supplemental Materials Table SI). The reverse primer annealed to 3’ sequences of spoIVB so that a TTA stop codon was placed after codons 391 (Δ34), 401 (Δ24), 409 (Δ16), and 416 (Δ9) in the spoIVB ORF. Wild-type or spoIVBSA378 alleles were incorporated into the deletions by using either PY79 (spo+) or SC2080 (spoIVBSA378) templates for PCR amplification (Supplemental Materials Table SI). To verify constructions the cloned templates were sequenced in their entirety.

Construction of B. subtilis Strains Carrying Modified spoIVB Genes

Deletions—to construct B. subtilis strains carrying the spoIVB COOH-terminal deletions, Δ34, Δ24, Δ16, and Δ9, two oligonucleotide primers were used to amplify a truncated spoIVB product using B. subtilis chromosomal DNA of strain PY79. The forward primer, F1 (5’-TATTAAACCGTTGCGGACATCCGTTGCGTT-3’) annealed to nucleotides 146 to 127 upstream of the spoIVB start codon and carried an embedded HindIII site. For the 3’ end the reverse primers R34 3F (5’-CGGAGCTC- CTTACGACAGCGACCACGAGCTTCC-3’) and R24 (5’-CGGAGTACTTATTCGCTTGGCTGATTTAC-3’) and R16 (5’- CGGAGTAC- CTAGATTTACATGACTATT-3’) and R9 (5’- CGGAGTACTTTAGATTACGACGAGCTGACATCGCC-3’) were used and each carried an embedded BamHI site. Each reverse primer would introduce a TAA stop codon at positions 418 (R9), 411 (R16), 403 (R24), and 393 (R34). The PCR amplified products were cleaved with HindIII and BamHI and cloned into pBluescript II KS (+). The clone was verified by sequencing and then subcloned into pDG364. pDG364 enables insertion of cloned DNA, in trans, at the amyl locus by a double crossover marker replacement (26). For each deletion the pDG364 clone was introduced into cells of SC1836 (spoIVB::apc) by DNA-mediated transformation and selection for chloramphenicol resistance (encoded by pDG364). Strains were TD776 (amy::spoIVB::spc), TD1034 (amy::spoIVB::spc), TD1036 (amy::spoIVB::spc), TD1038 (amy::spoIVB::spc), and TD775 (amy::spoIVB::spc).

Site-specific Mutations—A PCR-based mutagenesis method using double strand DNA template and selecting mutants with DpnI (22) was used to generate site-specific mutations at Thr939 (Thr to Ile and Thr to Asn) and Val1200 (Val to Glu) within the spoIVB ORF. The spoIVB template in pBluescript II KS (+) vector was used as a template for mutagenesis. The mutated templates were checked for the presence of the desired mutation and then subcloned into pDG364 before introduction of the mutated spoIVB gene into the chromosome of SC1836 (spoIVB::apc) at the amyl locus by a double crossover marker replacement (26) to generate the following strains, TD1160 (amy::spoIVBSA379I spoIVB::apc), TD1164 (amy::spoIVBSA379N spoIVB::apc), and TD1168 (amy::spoIVBSA379E spoIVB::apc).

Glutathione S-Transferase (GST) Pull-down Assays

The GST pull-down assay was carried out using immunoblotting to detect protein partners as described in Ref. 22. To alleviate problems associated with membrane-bound proteins, purified proteins were used. Incubation mixtures consisted of 50 µl of prepared glutathione-Sepharose beads, 50 µg of PDZ protein (His tag purified) mixed with either binding buffer only (20 mM Tris-Cl, 200 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 50 µg of GST protein in binding buffer or 50 µg of GST-fusion protein in binding buffer. Binding reactions were incubated for 4 h at 4 °C; then the beads were washed three times with binding buffer using a refrigerated microcentrifuge (4 °C). Bound complexes were eluted with reduced glutathione (20 µM in 50 mM Tris-Cl, pH 8.0) and analyzed by 15% SDS-PAGE, followed by immunoblotting with an antibody raised to a chimeric GST-PDZ protein (see “Deletions”). Experiments were performed at least three times. Molecular masses are shown in kDa. Assays were as described in the legend to Fig. 6. The following reagents were used.

GST–IVB°C—A truncated SpoIVB polypeptide consisting of the COOH-terminal 41 residues of SpoIVB fused to the GST protein. The spoIVB ORF from codon 385 to the stop codon was amplified using the primers BofA—CCCGTGCACATCCATTCGTTC (5’-CGGAGCTC- CTTACGACAGCGACCACGAGCTTCC-3’) and IVBN (5’- TCCCCCCGGCGTACGTCGTTTTTCTTTTCCATAAAATC-3’), which included BamHI and Xmal sites to facilitate cloning into the pGEX-4T-3 vector and in-frame fusion to the 3’-end of GST carried in pGEX-4T-3. The recombinant plasmids were checked for errors by nucleotide sequencing and expression of the polypeptides were confirmed by isopropyl-1-thio-β-D-galactopyranoside induction in BL21(DE3)- pLys-S cells and SDS-PAGE analysis.

GST–IVB°C (Δ9), GST–IVB°C (Δ16), and GST–IVB°C (Δ24)—These were identical to GST–IVB except that the COOH-terminal SpoIVB polypeptides carried mutations at codons 393 (Thr to Ile and Thr to Asn) and 395 (Val to Glu).

GST–IVB—A truncated SpoIVB polypeptide consisting of the NH-terminal 32 residues of SpoIVB fused to the GST protein. The spoIVB ORF from codon 385 to codon 416 was amplified using the primers BofA—CCCGTGCACATCCATTCGTTC (5’-CGGAGCTC- CTTACGACAGCGACCACGAGCTTCC-3’) and IVBN (5’- TCCCCCCGGCGTACGTCGTTTTTCTTTTCCATAAAATC-3’), which included BamHI and Xmal sites to facilitate cloning into the pGEX-4T-3 vector and in-frame fusion to the 3’-end of GST carried in pGEX-4T-3. The recombinant plasmids were checked for errors by nucleotide sequencing and expression of the polypeptides were confirmed by isopropyl-1-thio-β-D-galactopyranoside induction in BL21(DE3)-pLys-S cells and SDS-PAGE analysis.

GST–Δbofa—This was a fusion of GST at its COOH terminus to residues 58–67 of BofA. The bofa ORF (beginning at codon 58 and ending at the stop codon) was PCR amplified by the primer pair BofA-V58-F (CCGGAGCTCCTGGCGGATTCGAGCTTCC-3’) and BofA-R (CCGCTCGAGTTAAAGCTTTATGCTATGACGCG). These primers carried the BamHI and Xmal sites (underlined) to facilitate cloning into the pGEX-4T-3 vector and fusion to the 3’-end of GST. The recombinant
Antibodies—Because of the small size of the PDZ domain protein (10 kDa) we chose to generate an antisera in rabbits to a hybrid protein consisting of PDZ fused to GST. The GST coding region on the pGEX-4T-3 vector (Amersham Biosciences) was amplified by the primer pair FA-Q100-F (5'-CTCGAGTGTTCTTCCTGATTTAAAAGTTATACCTGGC) and IVBR, which included BamHI and XhoI sites to facilitate cloning into pET28b(+). The recombinant plasmids were checked for errors by nucleotide sequencing and expression of the polypeptides was confirmed by isopro-}

\[\text{FIG. 1. Autoproteolysis of SpoIVB. A schematic representation of SpoIVB is shown with the functionally important domains illustrated. The upper figure shows the NH}_2\text{-terminal region in more detail and the NH}_2\text{-terminal positions of truncated SpoIVB expression clones used in this work. Self-cleavage cis and trans reactions occurring at the NH}_2\text{-terminus are shown. The lower section shows the PDZ and serine peptidase domains as well as the catalytic His, Asp, and Ser residues of the serine peptidase domain. The COOH-terminal residues of the four COOH-terminal deletions used in this study are also shown. Numbers refer to amino acids with SpoIVB.}\]

\[\text{Plasmids were validated as described for GST-B385. GST-FA264 and GST-FA189—This was a fusion of GST at its COOH terminus to residues 100–264 or 100–189 of SpoIVFA. The spoIVFA ORF was amplified by the primer pair FA-9100-F (5'-CCGGGATCCACGATTAAACCCGCCG) and FA-V100-R for GST-IVFA(100–189) (5'-CCCGTCGAGTTAGTACCTCAACCATGAC) or FA-R for GST-IVFA(100–264) (5'-CCGGTCGAGTTATCTCAAGAATACCCTG), which included BamHI and XhoI sites, respectively, to facilitate cloning into pGEX-4T-3 vector and in-frame fusion to GST. The recombinant plasmids were validated as described for GST-B385.}\]

\[\text{PDZ-His—The spoIVB ORF from codon 98 to 193, corresponding to the PDZ domain, was amplified using the primer pair PDZF (5'-CATGGCCCGTCCAGTTCTTCTCTCGT) and PDZR (5'-CCGGTCGAGCAGCTGTTGGTTTGGTTTAC), which included NcoI and XhoI sites, respectively, to facilitate cloning into pET28b(+)) vector and COOH-terminal fusion to the GST coding region on the pGEX-4T-3 vector (Amersham Biosciences) and SDS-PAGE analysis.}\]

\[\text{RESULTS}\]

\[\text{Autoproteolysis of SpoIVB—Full-length SpoIVB (SpoIVBM1; 46 kDa) and NH}_2\text{-terminal deletions of residues 1–35 (SpoIVBM36; 42 kDa), 1–52 (SpoIVBN53; 40 kDa), 1–62 (SpoIVBF63; 39 kDa), and 1–74 (SpoIVBT75; 38 kDa) were expressed from pET vectors using a membrane-free, in vitro transcription-translation system and [\text{35S}]Met labeling. For simplicity we will henceforth refer to these clones as M1, M36, N53, F63, and T75, respectively (Fig. 1). All ORFs were placed under the control of the pET promoter, ribosome binding site, and an ATG start codon (see “Experimental Procedures”). M36 (described as IVBΔ), which has the NH}_2\text{-terminal signal sequence deleted has previously been shown to be proteolytically active and can cleave another SpoIVB template (proteolytically inactive M36-S378A or M36*) in trans (15). The N53, F63, and T75 proteins were designed to correspond to the individual polypeptides identified from NH}_2\text{-terminal sequence analysis of purified SpoIVB cleavage products (15). Expression of full-length SpoIVB protein, M1, produced three products of 46, 40, and 38 kDa (Fig. 2A). Expression of the truncated spoIVB ORF's produced bands of 42, 40, 39, and 38 kDa for M36; bands of 40, 39, and 38 kDa for N53; bands of 39 and 38 kDa for F63; and a
Reactions were stopped and fractionated on SDS-PAGE gels. Molecular masses are shown in kDa. Polypeptides, M36, N53, F63, and T75 were expressed using an in vitro expression experiment and the reaction stopped prior to mixing. Both proteins were co-incubated for 1 h at 37 °C before fractionation on SDS-PAGE gels. Molecular masses are shown in kDa.

![Image](https://example.com/fig2.png)

**Fig. 2. In vitro expression of SpoIVB polypeptides.** A, pET28b(+) clones encoding full-length (M1) or NH2-terminally deleted SpoIVB polypeptides, M36, N53, F63, and T75 were expressed using an in vitro transcription-translation system for 1 h at 37 °C in the presence of [*35S*]Met. Reactions were stopped and fractionated on SDS-PAGE gels. B, in vitro expression experiments were as described in A but pET28b(+) clones encoded equivalent SpoIVB proteins (labeled with *), but carried the S378A allele that inhibits self-processing. C, mixing experiments were performed by incubating a [*35S*]Met-labeled template with an equal volume of an unlabeled SpoIVB protein. In each case proteins were produced from an in vitro expression experiment and the reaction stopped prior to mixing. Both proteins were co-incubated for 1 h at 37 °C before fractionation on SDS-PAGE gels.

Single species of 38 kDa for T75 (Fig. 2A). Interestingly, for M36 the slowest migrating band of 42 kDa was less abundant than the 40- and 38-kDa species. Similarly, for both M36 and N53 the 39-kDa band was of low abundance while in full-length IVB it was not observed at all. In each case the slowest migrating band corresponded to the predicted molecular weight of the encoded SpoIVB protein and this was confirmed by performing an identical analysis using the same clones engineered to carry a mutation (S378A; Ser to Ala) in the catalytic serine at position 378 (termed M1*, M36*, N53*, F63*, and T75*). Expression of these clones showed that, with one exception, mutation of the active site serine prevented cleavage (Fig. 2B), confirming that cleavage is autoproteolytic. Minor bands were seen that could correspond to cleavage products (notably F63*) and we believe these may result from low levels of residual activity that has been shown to occur despite the presence of the S378A mutation (16). The one discrepancy was with expression of the full-length SpoIVB protein (M1*) carrying the S378A mutation where a secondary band of 42 kDa was detectable. Residual proteolytic activity within the catalytic triad might account for this but it does not explain why proteolysis did not proceed further nor likewise in the case of the M36*, N53*, or F63* polypeptides. As proposed previously, it is more likely that codon 36 (methionine) of the spoIVB ORF provides a secondary start site for translation in the in vitro system used here (15).

Cleavage Reactions: Cis or Trans?—To determine the ability of SpoIVB to cleave in trans we mixed unlabeled SpoIVB products from an in vitro transcription and translation system with [*35S*]Met-labeled SpoIVB templates that carried the S378A allele, and thus, were unable to autonomously self-process (as shown in Fig. 2B). As shown in Fig. 2C all four truncated versions of SpoIVB, M36, N53, F63, and T75, were able to cleave the labeled M36* protein carrying the S378A allele, producing, in each case, a 40-kDa cleavage product corresponding in size to the first cleavage site (between residues 52 and 53). As a control we also used an unlabeled M36* protein and showed that this failed to cleave M36*. One further reaction performed here was to examine trans cleavage using a SpoIVB protein (V100) in which residues 1–99 had been deleted from the NH2 terminus. In other work this truncated SpoIVB protein was shown to cleave SpoIVF in trans using a similar mixing experiment (21). We observe here that V100 was also able to cleave M36* in trans.

In contrast to the use of M36* as a template neither the N53* and T63* proteins (both carrying the S378A allele) were cleaved by any of the five active SpoIVB forms, M36, N53, F63, T75, or V100 (Fig. 2C). These results show that the M36 protein can serve as a template for trans cleavage but not the N53 or F63 proteins. Because expression of M36, N53, or F63 lead to self-proteolysis ultimately generating a 38-kDa species equivalent in size to T75 we conclude that the initial self-processing reaction of SpoIVB is in trans and subsequent cleavages must be in cis (Fig. 1).

Activity of Full-length SpoIVB—In the in vitro experiments shown in Fig. 2A expression was allowed to proceed for 1 h. Prolonged in vitro expression of either full-length M1 or M36 revealed that the full-length M1 protein self-processed at a much slower rate than the truncated M36 species (Fig. 3, A and B, respectively). With M36, little, if any of the 42-kDa species was detectable after 2 h of incubation and by 4 h self-processing appeared almost complete with the accumulation of stable 38- and 39-kDa species. In contrast, the 46-kDa M1 protein was still readily detectable after 8 h of incubation. However, additional species of 40, 39, and 38 kDa were also present. We
tested whether the full-length SpoIVB protein M1 was active as a peptidase using in vitro expression of M1 and mixing with appropriate 35S-labeled substrates as shown in Fig. 3. Expression of M1 alone generated a predominant 46-kDa species and lower molecular mass products of 40, 39, and 38 kDa (in agreement with Fig. 2A). Expression of M1* carrying the S378A allele abolished these lower molecular weight species. When the M1 active protein was mixed with the inactive labelled M1 template, M1*, no cleavage of M1* was detected. Similarly, M1 was unable to cleave the M36*, N53*, or F63* inactive species. We conclude that the full-length SpoIVB protein, M1, cannot cleave M36 in trans in contrast to M36 which can (see Fig. 2A). Expression of these proteins from pET expression plasmids in an in vitro system showed that M36(Δ9) appeared to be proteolytically active and generated self-cleavage products indistinguishable from expression of M36 alone (Fig. 4B). In contrast, the M36(Δ16), M36(Δ24), and M36(Δ34) proteins all appeared inactive and failed to undergo self-cleavage (Fig. 4B). Next, we made the same deletions but engineered these to encode SpoIVB proteins carrying the S378A allele and so were proteolytically inactive (Δ9*, Δ16*, Δ24*, and Δ34*). When expressed alone they produced only one product that could not undergo self-cleavage (Fig. 4, C–F). These proteins were expressed in vitro with [35S]Met labeling and then mixed with the unlabeled M36 protein (expressed, in vitro, in a separate reaction). Mixture of M36 with these COOH-terminal deletions showed that M36 could cleave the Δ9* (Fig. 4C), Δ16* (Fig. 4D), and the Δ24* proteins (Fig. 4E) but could not cleave the Δ34* protein in trans (Fig. 4F). In each case trans cleavage generated only one cleavage product corresponding to the cleavage site between residues 52 and 53 of the SpoIVB protein.

To confirm that proteolytic activity in the mutant proteins (Δ34, Δ24, and Δ16) was abolished we performed additional mixing experiments as shown in Fig. 5. Using a [35S]Met-labeled M36* SpoIVB protein (carrying the S378A mutation) as a template, we co-incubated with M36 or each of the four COOH-terminal deletions. As shown in Fig. 5 only M36(Δ9) was able to cleave the M36* template in trans. In a second

**Effect of COOH-terminal Deletions of SpoIVB on Self-cleavage**—Proteases carrying PDZ domains can target substrates using specific recognition motifs although the site of PDZ recognition is not necessarily the site of cleavage (27–29). Accordingly, we made four COOH-terminal deletions of the M36 protein (see Fig. 4A), M36(Δ34) carrying a deletion of residues 393 to 426 of the COOH terminus, M36(Δ24) a deletion of residues 403–426, M36(Δ16) a deletion of residues 411–426, and M36(Δ9) with a deletion of residues 418–426. Expression of these proteins from pET expression plasmids in an in vitro system showed that M36(Δ9) appeared to be proteolytically active and generated self-cleavage products indistinguishable from expression of M36 alone (Fig. 4B). In contrast, the M36(Δ16), M36(Δ24), and M36(Δ34) proteins all appeared inactive and failed to undergo self-cleavage (Fig. 4B). Next, we made the same deletions but engineered these to encode SpoIVB proteins carrying the S378A allele and so were proteolytically inactive (Δ9*, Δ16*, Δ24*, and Δ34*). When expressed alone they produced only one product that could not undergo self-cleavage (Fig. 4, C–F). These proteins were expressed in vitro with [35S]Met labeling and then mixed with the unlabeled M36 protein (expressed, in vitro, in a separate reaction). Mixture of M36 with these COOH-terminal deletions showed that M36 could cleave the Δ9* (Fig. 4C), Δ16* (Fig. 4D), and the Δ24* proteins (Fig. 4E) but could not cleave the Δ34* protein in trans (Fig. 4F). In each case trans cleavage generated only one cleavage product corresponding to the cleavage site between residues 52 and 53 of the SpoIVB protein.

**Fig. 3. Expression of full-length SpoIVB.** The M1, full-length (46 kDa), SpoIVB protein (A), and the M36 (42 kDa) SpoIVB protein (B) were expressed in vitro using [35S]Met labeling. Aliquots were removed at appropriate time points and fractionated on SDS-PAGE gels. C, mixing experiments were performed using an unlabeled, full-length, M1 protein produced from an in vitro expression experiment and [35S]Met-labeled templates. Co-incubation of M1 and the labeled template was for 1 h, after which the products were fractionated on SDS-PAGE gels. Molecular masses are shown in kDa.

**Table 3.**

| labelled template | M1 | M1* | M36* | N53* | F63* |
|------------------|----|-----|------|------|------|
| Unlabelled M1    | -  | -   | +    | -    | +    |
|                  | 46 | 42  | 40   | 39   | 38   |
SpoIVB Transmembrane Signaling

Fig. 4. COOH-terminal deletion analysis. A, the four COOH-terminal deletions of SpoIVB made in this study are shown (only the COOH-terminal region is shown). The deletions are placed above a protein sequence alignment of the corresponding COOH termini of SpoIVB homologues aligned using Clustal W (40). The catalytic serine at position 378 is shown and the THV amino acids conserved among all SpoIVB homologues and is similar to the PDZ binding motif (T/S)(X)F(V/M/L)M is shaded. Abbreviations and accession numbers are: Bautb, B. subtilis strain 168 (accession number NP 390303, residues 368–425); Bautm, Bacillus anthracis, strain Ames (accession number NP 846625, residues 374–432); Banta, B. anthracis, strain A2012 (accession number NP 658209, residues 371–429); Beere, Bacillus cereus, strain ATCC14579 (accession number NP 833887, residues 374–432); Btea, Bacillus stearothermophilus, strain 10 (residues 372–431); Bhao, Bacillus halodurans (accession number NP 243641, residues 379–437); Cltet, Clostridium tetani, strain E88 (accession number NP 782180, residues 351–402); Cibot, Clostridium botulinum, strain ATCC352O (residues 355–408); Clace, Clostridium acetobutylicum, (accession number NP 348991, residues 345–395); Ciper, Clostridium perfringens (accession number NP 562730, residues 337–386); Cuth, Clostridium thermocellum, strain ATCC 27405 (accession number ZP 90060568, residues 389–449); Cidif, Clostridium difficile (accession number AAB05660, residues 312–352); Oceba, Oceanobacillus iheyensis, strain ClOT1 (accession number NP 892784, residues 342–401); Theten, Thermotoga tengcongensis (accession number NP 622923, residues 369–428). B, in vitro expression is shown of the SpoIVB [35S]-Met-labeled COOH-terminal deletions M36(Δ9), M36(Δ16), M36(Δ24), and M36(Δ34) as well as M36 alone. Also shown are mixing experiments where the M36(Δ9) and M36(Δ16) (D), M36(Δ24) (E), and M36(Δ34) (F) proteins, each carrying the S378A mutation, were expressed and labeled with [35S]methionine (underlined) and then mixed for 1 h with an unlabeled M36, proteolytically active, in vitro expression product. The arrows show the observed cleavage products. Molecular masses are shown in kDa.

Fig. 5. Proteolytic activity of COOH-terminal deletions. In this experiment in vitro expression was used to produce a labeled M36* protein (carrying the S378A mutation). This was used as a template to measure proteolytic activity of the COOH-terminal deletions. The deletions were expressed from pET28h (+) plasmids and products were unlabeled. Co-incubation was allowed to proceed for 1 h before fractionation on SDS-PAGE gels. As a control M36, carrying no deletion, was also used to demonstrate cleavage. Molecular masses are given in kDa.

We next examined the sporoation and signaling phenotypes of these COOH-terminal deletions in B. subtilis. Isogenic strains were made that contained a modified spoIVB gene with the entire 5’-region intact but carrying the appropriate 3’-deletion. These constructs would encode a protein beginning at Met1 and therefore able to target and transit the IFM. As shown in Table I sporulation was completely abolished in mutants carrying the COOH-terminal deletions of 16, 24, and 34 residues and was essentially no different from a spoIVB null mutant (SC1836) or a mutant unable to self-process (SC2080). In contrast, a strain carrying the 9-amino acid COOH-terminal deletion was unimpaired and formed heat-resistant, phase bright, spores. We also examined signaling of pro-5 processing in these mutants and confirmed that no gene expression occurred in the Δ16 (TD1034), Δ24 (TD1036), and Δ34 (TD775) mutants. Signaling in the Δ9 mutant (TD776) was normal and occurred at the same time and at the same level as in wild type cells.

Finally, we examined SpoIVB synthesis and autoproteolysis during sporulation (Supplementary Materials Fig. S1). In wild
type cells (strain PY79), the full-length 46-kDa species was processed beginning 2.5 h following the onset of sporulation. As has been shown previously, SpoIVB autoproteolysis is rapidly processed generating intermediate cleavage products that are then processed further to inactive forms (15). In SC2080 cells carrying the spoIVB378 allele, SpoIVB is cleaved directly to the inactive forms without the appearance of the intermediate species. The fully processed forms of SpoIVB (in Supplemental Materials Fig. S2 these run as one band of approximately 36 kDa) are considered inactive because first, signaling does not occur in SC2080 cells (15) and, in vitro, M36H cannot cleave SpoIVFA (21). To account for cleavage in SC2080 cells it has been proposed that SpoIVB is subject to proteolysis by a second protease (15). Because the V100 form of SpoIVB is proteolytically active (see Fig. 3C) this secondary cleavage must occur at COOH-terminal sites. In TD776 cells that carried the 9-amino acid COOH-terminal deletion somewhat lower levels of SpoIVB were detectable yet the same temporal profile of cleavage products as in spo+ cells was discernable suggesting that autoproteolysis was normal. In contrast, cells carrying the 24- or 34-amino acid COOH-terminal deletions produced essentially no SpoIVB protein. In the case of the spoIVB316 mutant the full-length SpoIVB protein was detectable but no cleavage was observed. This deletion analysis showed that deletion of more than 9 COOH-terminal residues of SpoIVB eliminates its protease activity and drastically reduces the steady state level of the protein in vivo. This is most probably a consequence of decreased stability rendering it much more susceptible to proteolytic breakdown. In addition, for SpoIVB to serve as a substrate, deletion of more than 24 COOH-terminal residues prevents trans cleavage and we infer that residues between 392 and 402 are important for recognition of SpoIVB as a proteolytic substrate.

In Vitro Determination of SpoIVB Interactions—SpoIVB has the potential to undergo interaction with itself as well as with other protein partners and at least some of these interactions might utilize its PDZ domain. Two modes of interaction involving only SpoIVB can be envisaged. First, for which evidence has been presented above, SpoIVB could be maintained as azymogen by an intramolecular interaction involving its NH2 terminus. Second, the trans cleavage reaction that would release SpoIVB from the IFM following membrane translocation could be mediated by the PDZ-mediated recognition of SpoIVB as a substrate. At least three further examples of interactions have been proposed (18, 21). An interaction with BofC may inhibit the signaling role of SpoIVB and an interaction with SpoIVFA may permit SpoIVB-mediated cleavage of SpoIVFA resulting in the proposed disassembly of the pro-oK proteolytic complex in the OFM and release of SpoIVFA-mediated inhibition of pro-oK processing. Finally, it is also possible that SpoIVB interacts with BofA because this protein is essential for inhibiting the pro-oK processing complex.

To directly address which proteins could interact with the SpoIVB PDZ domain we used a GST pull-down assay (Fig. 6). Two GST hybrid proteins were constructed, GST-IJVN, where GST was fused at its COOH terminus to the NH2 terminus of SpoIVB (residues 1–32), and GST-IVKB, where GST was fused at its COOH terminus to the COOH-terminal 41 residues of SpoIVB (residues 385–426). The purified chimeric GST chimeras were co-incubated with the V100SA378 protein for 2 h in the presence of glutathione-Sepharose beads that bind specifically to GST. V100SA378 consisted of residues 100–426 of SpoIVB carrying the entire PDZ domain and the serine peptidase domain, i.e. was proteolytically inactive because of the Ser to Ala mutation at residue 378. Proteins that had bound to the glutathione-Sepharose beads were eluted and analyzed by SDS-PAGE and immunoblotting using a polyclonal antiserum to SpoIVB. As shown in Fig. 6A the V100SA378 was able to bind to both GST-IVB and GST-IJVN (because it eluted as a 36-kDa protein from beads mixed with GST-IVB (lane 3) or GST-IJVN (lane 4) but not when mixed with GST alone (lane 2)), which demonstrated an interaction of SpoIVB residues 100–426 with the extreme NH2 and COOH termini of SpoIVB. To further refine the binding domain we used the 10-kDa SpoIVB PDZ domain in the binding experiments instead of V100SA378. As shown in Fig. 6B the PDZ domain bound to GST-IVB (lane 3) and GST-IJVN (lane 8) but failed to bind to the GST protein alone (lane 2). Using deletion analysis we have shown that residues 392–402 are important for autoproteolysis (Fig. 4) and the most likely explanation is that the PDZ domain binds to this region. This region contains a motif Thr393-His394-Val395 that matches the classical Ser/Thr-X-Val-COOH motif found at the extreme COOH terminus of PDZ-recognized proteins (29–31). In this case, the Thr-X-Val motif, although in the COOH-terminal region of the protein does not lie at the extreme COOH terminus. We made site-specific changes at Thr393 and Val395 in the GST-IJVN polypeptide and assessed their effect on binding to the PDZ domain. As shown in Fig. 6B, the GST-IVB(T393I) (lane 4), GST-IVBC(V395E) (lane 5), and GST-IVBC(T393N) (lane 6) changes each markedly reduced their interaction with the PDZ protein when compared with the GST-IVB polypeptide run in parallel (note that we have repeated these experiments two times with similar results). Thr393 and Val395 are therefore important for interaction with the PDZ domain and based on their similarity to known PDZ-recognition motifs could form an internally placed PDZ recognition domain. In support of this strains carrying spoIVB3793I, spoIVB3793N, and spoIVB395E mutations were phenotypically SpoIVB− and failed to make heat-resistant spores (Table I). In these strains SpoIVB did not self-process (not shown) and pro-oK processing did not occur.

Next, we asked whether the SpoIVB PDZ domain could interact with GST proteins fused to COOH-terminal segments of BofA (GST-3BofA, residues 58–87, Fig. 6, lane 7) and SpoIVFA (GST-IVFA(100–264), residues 100–264, Fig. 6, lane 10). We were unable to demonstrate binding of the PDZ domain to...
The Effect of the PDZ Domain on Self-cleavage of SpoIVB—
The spoIVBD149N mutation was introduced into the pET vectors carrying spoIVB templates to encode, full-length SpoIVB (M1\(^B\)) and the truncations M36\(^D\), N53\(^D\) F63\(^D\), and T75\(^D\). D149N is an Asp to Asn change at position 149 within SpoIVB and is the most conserved residue of the PDZ domain (18). In B. subtilis, spoIVBD149N cells are temperature-sensitive for sporulation and at the restrictive temperature they fail to make heat-resistant spores and cause impaired self-cleavage of SpoIVB with a corresponding delay in signaling of pro-\(\alpha\) processing (18, 21). In vitro expression of SpoIVB templates carrying the D149N mutation was examined by \(^{[35}\text{S}]\text{Met}\) labeling (Supplementary Materials Fig. S3A). Expression of full-length M1\(^B\) was similar to expression of M1 alone (see Fig. 2A) but with reduced amounts of the 40- and 38-kDa species. Expression of M36\(^D\) showed an accumulation of the 42-kDa species and an absence of the 39-kDa species but was otherwise similar to M36 expression (Fig. 2A). N53\(^D\) expression was similar to N53 expression (see Fig. 2A) but the 39-kDa species was absent. F63\(^D\) expression appeared aberrant and we could not discern whether one or two species were expressed (in contrast to expression of F63, Fig. 2A). T75\(^D\) expression was identical to T75. Extended expression of M36\(^D\) (Supplementary Materials Fig. S3B) showed that after 8 h of expression there was a marked difference in self-cleavage when compared with extended expression of M36 (see Fig. 4B). First, the 42-kDa M36 product was more slowly self-processed and was still present 8 h after incubation. Second, we could not detect the 39-kDa cleavage product. Third, a number of new cleavage products of

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**Fig. 6. SpoIVB-SpoIVB interactions.** The GST pull-down assay was carried out as described under “Experimental Procedures.” Incubation mixtures consisted of glutathione-Sepharose beads, PDZ protein (His-tag purified) mixed with either binding buffer only, GST protein in binding buffer, or GST fusion protein in binding buffer. GST-IVB(100–264)–GST-IVB(100–264). The COOH-terminal segments of these proteins were selected because they are both located in the compartment where SpoIVB acts (between the IFM and OFM) and because PDZ domains normally interact with the COOH termini of proteins. We have also performed a similar experiment but using a GST-IVFA(100–189) protein comprising the COOH-terminal region that has been proposed elsewhere (21) to bind to the PDZ domain (residues 102–186) of SpoIVB and also failed to detect binding to the PDZ (not shown). As a further test we also showed that neither the GST-IVFA(100–189) nor GST-IVFA(100–264) proteins could bind to the V100SA378 protein (data not shown). In contrast, we were able to demonstrate clear binding of the SpoIVB PDZ domain to the extreme COOH terminus of BofA (lane 7). Finally, we examined binding of the PDZ domain to BofC (lane 9) and failed to detect interaction. Although our failure to detect binding using only one method is not unequivocal proof that two proteins do not interact, the fact that we can reliably detect binding to the NH\(_2\) terminus of SpoIVB, to the COOH terminus of SpoIVB, and to BofA does suggest that the PDZ domain does not interact with either BofC or SpoIVFA.

The Effect of the PDZ Domain on Self-cleavage of SpoIVB—

The spoIVBD149N mutation was introduced into the pET vectors carrying spoIVB templates to encode, full-length SpoIVB (M1\(^B\)) and the truncations M36\(^D\), N53\(^D\) F63\(^D\), and T75\(^D\). D149N is an Asp to Asn change at position 149 within SpoIVB and is the most conserved residue of the PDZ domain (18). In B. subtilis, spoIVBD149N cells are temperature-sensitive for sporulation and at the restrictive temperature they fail to make heat-resistant spores and cause impaired self-cleavage of SpoIVB with a corresponding delay in signaling of pro-\(\alpha\) processing (18, 21). In vitro expression of SpoIVB templates carrying the D149N mutation was examined by \(^{[35}\text{S}]\text{Met}\) labeling (Supplementary Materials Fig. S3A). Expression of full-length M1\(^B\) was similar to expression of M1 alone (see Fig. 2A) but with reduced amounts of the 40- and 38-kDa species. Expression of M36\(^D\) showed an accumulation of the 42-kDa species and an absence of the 39-kDa species but was otherwise similar to M36 expression (Fig. 2A). N53\(^D\) expression was similar to N53 expression (see Fig. 2A) but the 39-kDa species was absent. F63\(^D\) expression appeared aberrant and we could not discern whether one or two species were expressed (in contrast to expression of F63, Fig. 2A). T75\(^D\) expression was identical to T75. Extended expression of M36\(^D\) (Supplementary Materials Fig. S3B) showed that after 8 h of expression there was a marked difference in self-cleavage when compared with extended expression of M36 (see Fig. 4B). First, the 42-kDa M36 product was more slowly self-processed and was still present 8 h after incubation. Second, we could not detect the 39-kDa cleavage product. Third, a number of new cleavage products of
30, 31, and 32 kDa were present and these accumulated as the length of expression increased. We conclude that the D149N PDZ mutation impairs but does not block self-cleavage of SpoIVB.

**DISCUSSION**

PDZ domains are being shown to play important roles in signaling processes. For example, the *Drosophila* protein INAD carries five PDZ domains that together form a scaffold for the G-protein-coupled phototransduction cascade in the fly eye (32). Another well studied example is the post-synaptic density protein (PSD-95) that has three PDZ domains and is involved in glutamate receptor clustering at synapses (33, 34). An emerging theme is the role of PDZ domains in transmembrane signaling and regulated proteolysis. In *E. coli*, in response to a heat shock, the COOH termini of misfolded or unfolded outer membrane porins in the periplasm bind to the DegS protease and activate it, which in turn leads to the cleavage of a transmembrane protein, RseA, that inhibits the σE transcription factor. Upon release from this inhibition, σE orchestrates expression of stress genes (35). DegS is thought to exist normally in an equilibrium between an active and inactive form and inhibition occurs when its PDZ and protease domains interact in cis. Under stress conditions, the COOH terminus of outer membrane porins can bind to the DegS PDZ domain, stabilize it in the active form and prevent PDZ binding in cis.

In this work we have dissected a similar pathway of regulated processing being driven by multiple interactions using a single PDZ domain. In SpoIVB signaling of pro-σK processing (see Fig. 7), the 46-kDa SpoIVB protein must activate proteolysis of the transcription factor SpoIVB, and in the OFM and is complexed to SpoIVFA and SpoIVFB. Binding to BofA enables SpoIVB to cleave the COOH terminus of SpoIVFA between residues 145 and 175. Step 5, removal of the COOH terminus of SpolIVFA disrupts the complex because the COOH terminus of SpoIVFA is necessary to recruit and stabilize all three partners in the OFM. Release of BofA-SpolIVFB contact activates the SpoIVFB zinc-metalloprotease and leads to cleavage of pro-σK.

![Fig. 7. PDZ-mediated interaction in activation of the pro-σK processing complex.](image-url) Step 1, SpoIVB (46 kDa) is synthesized in the forespore compartment under control of EσF and then 1 h later by EσG. During the early EσF phase spoIVB expression is repressed but low levels of SpoIVB are still produced. Signaling does not occur because of the competitive action of the BofC. In our model BofC (EσF controlled) secretion occupies the secretion apparatus such that SpoIVB is unable to translocate the IFM. To maintain SpoIVB as a zymogen, the PDZ domain of SpoIVB binds to its NH2-terminal domain. Step 2, during the EσF phase of forespore gene expression there is a surge of spoIVB expression and a simultaneous decrease in bοfC transcription. This enables SpoIVB to target and be secreted across the IFM. As SpoIVB crosses the IFM it unfolds releasing the NH2-terminal inhibition of its proteolytic activity. Next, SpoIVB uses its PDZ domain to bind to the COOH terminus of an adjacent SpoIVB molecule and cleaves it between residues 52 and 53 releasing it from the IFM as a 40-kDa polypeptide species. Step 3, following release from the IFM SpoIVB undergos either one or two further self-cleavage reactions these being in cis. The final cleavage generates a stable 30-kDa polypeptide. Step 4, using its PDZ domain SpoIVB targets and binds to the COOH terminus (residues 58–87) of BofA that resides in the OFM and is complexed to SpoIVFA and SpoIVFB. Binding to BofA enables SpoIVB to cleave the COOH terminus of SpoIVFA between residues 145 and 175. Step 5, removal of the COOH terminus of SpolIVFA disrupts the complex because the COOH terminus of SpolIVFA is necessary to recruit and stabilize all three partners in the OFM. Release of BofA-SpolIVFB contact activates the SpolIVFB zinc-metalloprotease and leads to cleavage of pro-σK.
SpoIVB is secreted across the IFM, which renders it proteolytically active. Its first cleavage reaction is in trans and requires a PDZ-mediated recognition of another SpoIVB protein tethered to the IFM. Following proteolytic release of SpoIVB from the IFM two further cis cleavage reactions occur after which SpoIVB uses its PDZ domain to target BofA. The recruitment of SpoIVB to the pro-αK processing complex in the OFM would enable SpoIVB to accumulate at the OFM interface and cleave SpoIVFA leading to disassembly of the complex and activation of the SpoIVB zinc metalloprotease that cleaves pro-αK. The important aspects of this signal transduction pathway are discussed in further detail below with reference to Fig. 7.

The Order of Self-cleavage—Previous work has identified three SpoIVB cleavage products that correspond to the N53, F63, and T75 proteins expressed here. By examining in vitro expression of these proteins we have shown in this work that (i) M36 is cleaved into three fragments corresponding to N53, F63, and T75, (ii) N53 is cleaved into two smaller proteins corresponding to F63 and T75, (iii) F63 is cleaved once to T75, and (iv) T75 is not cleaved further. An important observation was that when F63 was expressed, it appeared to accumulate and was only slowly converted to the T75 fragment (Fig. 2, lane F63) but when M36 and N53 were expressed the F63 fragment was much less abundant in comparison to the T75 fragment (Fig. 2, lanes M36 and N53). We infer that the direct processing of N53 to T75 occurs at a much faster rate than processing via F63 (Fig. 1). More importantly, we show here that two types of self-cleavage reaction occur. The first reaction to occur is a trans cleavage reaction between residues 52 and 53 and any active form of SpoIVB appears to be able to cleave at this site in trans. However, once the N53 species is produced it no longer serves as a substrate for trans cleavage and subsequent reactions must occur in cis. To achieve this SpoIVB must presumably assume an appropriate tertiary structure to enable self-recognition and cleavage. We have also defined the minimal size of SpoIVB and shown that deletion of residues 1–99 of SpoIVB do not interfere with its serine peptidase activity and agrees with an earlier study showing that the V100 SpoIVB protein can cleave SpoIVFA in trans but that deletions within the PDZ domain failed to permit processing (21).

Trans-cleavage—Using deletion analysis we have shown that for SpoIVB to cleave another SpoIVB molecule in trans it must target a specific region at the COOH terminus (residues 393–402) just downstream of the serine peptidase domain. This region carries a Thr393-His-Val395 motif identical to the classical PDZ recognition motif ((S/T)-X-V-COO⁻) normally found at the extreme COOH terminus of PDZ-recognized proteins (29, 36). Mutation of the Thr and Val residues showed a marked reduction in their ability to bind to the PDZ domain. Moreover, in vivo, mutation of these residues abolished the ability of SpoIVB to signal processing of pro-αK. We believe this represents strong evidence that to facilitate trans cleavage SpoIVB must first bind to a COOH-terminal recognition motif of TXV. Binding would allow one of the two SpoIVB molecules to cleave its bound partner between residues 52 and 53 producing the first cut. We have shown here that autoproteolysis is impaired in SpoIVB polypeptides carrying the temperature-sensitive D149N allele within the PDZ domain. Although this mutation has the most pronounced effect on the PDZ domain it is important to remember that this allele permits abnormal autoproteolysis and signaling in vivo (although signaling only occurs at the permissive temperature). We demonstrated in vitro that disruption of the PDZ domain allowed nonspecific cleavage of SpoIVB generating additional cleavage products. In vivo we would expect these abnormal cleavage products to be functionally active in signaling so long as no more than 100 residues of the NH₂ terminus is removed. The PDZ domain is most probably important for the initial recognition of a SpoIVB partner for trans cleavage because expression of a SpoIVB protein (M36K) carrying D149N lead to a slower appearance of the 42-kDa N53 species when compared with M36 expression suggesting that the PDZ mutation must be interfering with this primary event (trans cleavage between residues 52 and 53) and agrees with our work here showing PDZ recognition of motifs within SpoIVB. However, we cannot rule out the possibility that the appearance of the additional cleavage products in the D149N mutants of SpoIVB could arise if the PDZ domain is also important in the cis cleavage, ensuring cleavage at specific sites. It is clear that the SpoIVB PDZ domain appears capable of recognizing internally placed motifs and there is now a growing number of proteins where the recognition motif is internally placed, the only condition is that the motif is presented in a “loop” structure (37).

Role of the NH₂ Terminus—The full-length SpoIVB protein, M1, is proteolytically inactive and fails to cleave another SpoIVB protein in trans. SpoIVB then, is azymogen, requiring cleavage to be rendered active and residues 1–52 must be involved in inhibiting the proteolytic activity. Residues 1–23 of SpoIVB are hydrophobic and resemble a classical lipoprotein signal sequence (although this has never been proven). SpoIVB has been shown to translocate a phospholipid membrane and, of course, to signal in B. subtilis it must do so because genetic experiments have ruled out the possibility for additional signaling partners (13). Using an in vitro method we have shown that residues 1–32 of SpoIVB can bind to the PDZ domain of SpoIVB. This suggests then that in addition to its role in binding to a second SpoIVB molecule the PDZ domain is responsible for a second self-interaction (that could be in cis or in trans) maintaining itself in an inactive form as is also the case with DegS (35). Interestingly, in this NH₂-terminal region there is a SerX-X-Leu motif similar to the classical (S/T)X recognition motif, and in SpoIVB homologues from other Bacillus species this motif is SXV. It seems probable then that the PDZ domain enables SpoIVB to interact both in cis and in trans using a general PDZ-recognition consensus motif of (S/T)X(V/L).

We have shown above that BoFC does not interact with SpoIVB. In work not shown we have also failed to demonstrate that BoFC is a proteolytic substrate for SpoIVB using in vitro mixing experiments. If BoFC was a substrate for SpoIVB this would provide a means for competitive inhibition. One remaining possibility for how BoFC inhibits SpoIVB mediated signaling is interference with secretion of SpoIVB across the IFM because both proteins must cross the IFM and deletion of BoFC allows SpoIVB signaling (Fig. 7). For now this remains the only rationale explanation for the negative role of BoFC in the αK checkpoint.

Interaction with the Pro-αK Processing Complex—SpoIVB can cleave the COOH terminus of SpoIVFA between residues 145 and 175 (21). This region is exposed to the space between the OFM and IFM and a simple explanation would be that SpoIVB targets this COOH-terminal region for proteolysis in much the same way as is done by some proteases that use PDZ domains for substrate recognition (e.g. TspA protease (29)). The failure to demonstrate binding of a truncated form of SpoIVB to SpoIVFA appears to rule out this possibility. On the other hand we were able to show that the SpoIVB PDZ domain could target and bind to BoFA. BoFA is a small polypeptide (molecular mass 9 kDa) with over half the protein embedded in the OFM (Fig. 7). The segment that binds SpoIVB is also exposed to the space between the inner and outer forespore membranes. Interestingly, this region of BoFA carries a number of putative PDZ-
SpoIVB Transmembrane Signaling

recognition motifs, whereas no such motifs are found in the COOH terminus of SpoIVF. The binding of SpoIVB to BoA may therefore provide the mechanism by which SpoIVB interacts with the pro-αK processing complex? SpoIVF serves as the protein responsible for recruiting and assembling the heteroligomeric pro-αK processing complex consisting of SpoIVF, SpoIVFB, and BoA. As a heteroligomeric complex this structure is inactive so long as all three partners remain bound together (8). Binding of SpoIVB to BoA would allow the serine peptidase to come into sufficiently close proximity to the COOH terminus of SpoIVF to cleave it and this would be in keeping with the known function of PDZ domains in building complex macromolecular signaling structures. Thus, in addition to its role as the inhibitor of SpoIVFB activity, BoA also serves as a scaffold mediating the interaction between SpoIVB and SpoIVF. The demonstration that SpoIVB can cleave SpoIVF in vitro indicates that BoA is not essential for interaction between the two proteins. Unfortunately, it has so far proven impossible to express native BoA in an in vitro system (21) but we would predict it would significantly enhance the cleavage efficiency if the appropriate protein-protein interaction could be established in this system. These results show the versatility of the SpoIVB PDZ domain in enabling multiple interactions. It is intriguing then that there is potentially a second PDZ-containing serine peptidase, CtpB, that co-regulates the same intercompartmental signaling cascade activating αK (38). CtpB resembles SpoIVB in that it is secreted across a membrane into the space between the IFM and OFM and it also carries a PDZ domain preceding the serine peptidase domain. However, it differs in that CtpB is made in the mother cell chamber of the sporulating cell and is secreted across the OFM. The serine peptidase domain resembles that of the carboxyl-terminal processing protease family (e.g. E. coli Tsp). Finally, in contrast to SpoIVB, CtpB is not essential for processing of pro-αK because deletion of ctpB permits delayed processing. It is currently unclear how CtpB could modulate signaling but interaction with SpoIVB, SpoIVF, and/or BoA have been proposed. An interesting possibility is that a less stringent PDZ interaction (compared with SpoIVB) with one or more of these targets would allow CtpB to target them for proteolysis following interaction with SpoIVB. For example, following disassembly of the pro-αK processing complex, CtpB could target and degrade BoA.

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