Impact of Membrane Fusion and Proteolysis on SpoIIQ Dynamics and Interaction with SpoIIIAH*3

Shinobu Chiba1, Kristina Coleman, and Kit Pogliano2
From the Division of Biological Sciences, University of California San Diego, La Jolla, California 92093-0377

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The onset of engulfment-dependent gene expression during Bacillus subtilis sporulation requires the forespore membrane protein SpoIIQ, which recruits mother cell proteins involved in late gene expression to the outer forespore membrane. Engulfment activates the late forespore transcription factor σE, which produces high levels of the secreted SpoIVB protease that is required for activation of the late mother cell transcription factor σK. Engulfment also triggers the proteolytic cleavage of SpoIIQ, an event that depends on the SpoIVB protease but not on σE activity. To determine if SpoIVB directly cleaves SpoIIQ and to determine if this event participates in the onset of late gene expression, we purified SpoIVB, SpoIIQ, and SpoIVFA (another SpoIVB substrate). SpoIVB directly cleaved SpoIIQ at the same site in vitro and in vivo and cleaved SpoIVFA in at least three different locations. SpoIIQ cleavage depends on membrane fusion, but not on σE activity, suggesting that the ability of SpoIVB to cleave substrates is regulated by membrane fusion. We isolated SpoIVB-resistant SpoIIQ proteins by random mutagenesis of codons at the cleavage site and demonstrated that SpoIIQ processing is dispensable for spore formation and for activation of late forespore and mother cell gene expression. Fluorescence recovery after photobleaching analysis demonstrated that membrane fusion releases SpoIIQ from an immobile complex, an event that could allow SpoIVB to cleave SpoIIQ. We propose that this membrane fusion-dependent reorganization in the complex, rather than SpoIIQ proteolysis itself, is necessary for the onset of late transcription.

Endospore formation in Bacillus subtilis and its relatives depends on engulfment, a phagocytosis-like process that mediates a dramatic rearrangement of the sporangium from two adjacent daughter cells, to an endospore in which the forespore lies within the cytoplasm of the larger mother cell (see Fig. 1; reviewed by Refs. 1 and 2). Engulfment is critical for sporulation, because it allows spore assembly to occur in a protected environment. It also serves as a morphological checkpoint for activation of the late forespore and mother cell transcription factors σG and σK, respectively (reviewed by Refs. 1–4). The engulfment-forming bacteria must therefore have some mechanism to sense the completion of engulfment and to couple this morphological event to the onset of late gene expression.

The forespore transcription factor σE is the first to become active after engulfment, but it remains unclear how σE is held inactive during engulfment or activated after engulfment. More is known about the regulation of the second late transcription factor, σK, which becomes active in the mother cell (summarized in Fig. 1B and reviewed by Refs. 1–4). The σK factor is initially synthesized as an inactive pro-protein containing a hydrophobic leader sequence, which functions as a covalently attached anti-sigma factor (5). This leader sequence is removed by the intramembrane protease SpoIVFB, which cleaves pro-σK within the membrane to release active σK (6–8). This processing event shares many characteristics with Regulated Intramembrane Proteolysis (RIP),3 a widespread signal transduction mechanism in which extracellular signals are transduced to a protease that cleaves its substrate within the plane of the membrane to release an active transcription factor (reviewed by Refs. 9 and 10). As is typical of RIP systems, the intramembrane protease SpoIVFB is inactive until it receives an extracellular signal. In the case of SpoIVFB, the signal is the prior activation of the late forespore-specific transcription factor σG (11), which produces higher levels of the SpoIVB protease (12). This protease disrupts an inhibitory complex between the SpoIVFB intramembrane protease and two other proteins, SpoIVFA, which is necessary for complex assembly and localization (13), and BofA, which inhibits SpoIVFB activity (Fig. 1B) (7, 11, 13). BofA is likely the main inhibitor of the SpoIVFB intramembrane protease, because it can inhibit SpoIVFB activity in an ectopic expression system (7), and because its genetic inactivation allows σK to become active in the absence of σG activity and in the absence of the SpoIVB signal transduction protease (11). The SpoIVB protease cleaves SpoIVFA and relieves BofA-mediated inhibition of SpoIVFB (12, 14–16). This pathway results in the indirect coupling of σK activity to BofA.

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2 To whom correspondence should be addressed: Division of Biological Sciences, University of California San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0377. Tel.: 858-822-1314; Fax: 858-822-1431; E-mail: kpogliano@ucsd.edu.

3 The abbreviations used are: RIP, regulated intramembrane proteolysis; FRAP, fluorescence recovery after photobleaching; PBS, phosphate buffered saline; GFP, green fluorescent protein; AMS, 4-acetamido-4-dimethylstilbene-2;2′-disulfonic acid; KLH, Keyhole limpet hemocyanin; GST, glutathione S-transferase; BSA, bovine serum albumin; t1, t2, t3, . . . time of sporulation, with the number indicating hours after the initiation of sporulation by resuspension; FM, forespore membrane; OFM, outer forespore membrane.
engulfment, by virtue of its dependence on the prior activation of $\sigma^G$.

Recent evidence suggests that activation of $\sigma^K$ is also directly coupled to the completion of engulfment in two distinct manners (17). First, although the genetic elimination of BofA allows $\sigma^K$ to become active in the absence of $\sigma^G$ and SpoIVB, it does not allow $\sigma^K$ activity in engulfment defective mutants (11, 17). Thus, the failure of these mutants to activate $\sigma^G$ is not the only reason for the failure to activate $\sigma^K$. This suggests that $\sigma^K$ activation is directly governed by engulfment via a pathway that does not depend on BofA, the known inhibitor of SpoIVFB intramembrane protease activity. We therefore previously proposed the existence of two distinct checkpoints for $\sigma^K$ activity (17): the previously described forespore checkpoint that couples $\sigma^K$ activity to $\sigma^G$ activity via the BofA inhibitor (11) and the engulfment checkpoint that couples $\sigma^K$ activity to the completion of engulfment via a BofA-independent mechanism (Fig. 1B) (17). Second, engulfment also appears likely to govern the activity of the SpoIVB protease that participates in the forespore checkpoint. This was first suggested by the observation that SpoIVB is synthesized both before engulfment by the early forespore transcription factor $\sigma^G$ and after engulfment by the late forespore transcription factor $\sigma^G$ (18). Additional support for the regulation of SpoIVB protease activity came from observations that the forespore membrane protein SpoIIQ is subject to a proteolytic processing event that depends on both engulfment and on the SpoIVB protease (17, 19). However, SpoIIQ processing occurs with apparently identical kinetics in wild-type strain or in a strain with a mutation in the gene encoding $\sigma^G$ (spoIIQG). Thus, if SpoIVB directly cleaves SpoIIQ then its activity or access to substrates must be regulated by engulfment.

SpoIIQ proteolysis could be involved in engulfment-dependent gene expression, because this forespore membrane protein plays several key roles in this stage of sporulation. First, SpoIIQ is essential for the engulfment-dependent activation of $\sigma^G$ (20, 21), although its precise role in this process remains unclear. Second, SpoIIQ participates in engulfment, providing a secondary mechanism that is necessary for membrane migration when the activity of the primary engulfment machinery is compromised (22). This secondary engulfment mechanism requires the interaction between the extracellular domains of SpoIIQ and that of the mother cell membrane protein SpoIIAH (Fig. 1B) (22, 23). This interaction can readily be detected by several biochemical methods such as co-immunoprecipitation (23), affinity chromatography (23, 24), and sucrose density gradient analysis (Fig. 5). Third, SpoIIQ is required for the localization of mother cell membrane proteins needed for both $\sigma^G$ and $\sigma^K$ activation to the outer forespore membrane that is the site of intracellular signal transduction (17, 23, 24). Specifically, the interaction between SpoIIQ and SpoIIAH prevents SpoIIAH from diffusing away from the outer forespore membrane (23, 24), where it is required for $\sigma^G$ activity. SpoIIAH and SpoIIQ together are needed to localize the $\sigma^K$-processing machinery, SpoIVFA and SpoIVFB, to the outer forespore membrane, although it is unclear if SpoIIQ or SpoIIAH directly interact with SpoIVFA or SpoIVFB (17, 24). Interestingly, SpoIIQ, SpoIIAH, and SpoIVFB all localize to foci surrounding the forespore (Fig. 4B) (17, 19, 23). These foci might represent synapse-like sites for intracellular signal transduction, perhaps allowing transcription in both cells to be coordinately regulated by engulfment. It remains unclear how the completion of engulfment is sensed, and if SpoIIQ serves only as a scaffold to localize these signal transduction proteins or if it also participates in signal transduction.

**SpoIIQ proteolysis could affect its interaction with proteins involved in $\sigma^G$ and $\sigma^K$ activation, and therefore provides a potential mechanism by which late transcription might be coupled to engulfment. To determine if this is the case, and to determine if SpoIVB directly cleaves SpoIIQ, we further characterized SpoIIQ proteolysis. Specifically, we demonstrated that purified SpoIVB directly cleaves SpoIIQ, mapped the sites at which SpoIIQ is cleaved in vivo and in vitro, and isolated protease-resistant mutants. These mutants had no effect on $\sigma^G$ or $\sigma^K$ activity or spore formation, demonstrating that SpoIIQ processing is dispensable for sporulation. SpoIIQ proteolysis requires the membrane fusion event that is the final step of engulfment. Finally, fluorescence recovery after photobleaching (FRAP) demonstrates that the mobility of SpoIIQ dramatically increases after membrane fusion (in the absence of proteolysis), indicating that SpoIIQ is released from an essentially immobile complex after fusion. This reorganization in the SpoIIQ complex might control both proteolysis and signal transduction.**

**EXPERIMENTAL PROCEDURES**

*Strain and Plasmid Construction—B. subtilis* strains (Table 1) were constructed by transformation (25). Plasmids (supplemental Table S1) were constructed by standard cloning methods and site-directed mutagenesis (26). Following introduction into the *B. subtilis* chromosome, recombinants were checked for their antibiotic resistance, the inactivation of *amyE*, and the loss of any additional drug resistance markers on the plasmid backbone. Primers used for plasmid construction are in supplemental Table S2. Plasmid pH507 (amyE::PspoIIQgfp(Δ2–6)-spoIIQkat (19)) by site-directed mutagenesis (26) using a primer, SP1. Plasmids encoding either *spoIIQ*, *gfp*:: *spoIIQ*, or *gst*:: *spoIIQ* derivatives were constructed by site-directed mutagenesis using either pH505 (amyE::PspoIIQspoIIQ-gst::PspoIIQkat) or pH507 (amyE::PspoIIQkat) as the template. Primer SP2 was used for pH510 (amyE::PspoIIQkat) and the plasmids encoding *spoIIQ* cleavage site mutants (V72, G73, or K74) were constructed by using either SP3, SP4, or SP5 (respectively), which had a random mixture of bases in the target codon. pH550 (spoIIQΔ72) was also isolated by this random mutagenesis. The DNA sequence of the resulting products was confirmed prior to transformation into *B. subtilis*. Plasmids encoding mono-Cys mutants of *spoIIQ* were constructed by site-directed mutagenesis using the template plasmid pH505 (spoIIQ) and primers either SP10 (pCH528; A62C), SP11 (pCH530; D64C), SP12 (pCH531; D68C), SP13 (pCH532; V72C), SP14 (pCH533; E71C), SP15 (pCH534; V72C), SP16 (pCH535; G73C), or SP17 (pCH536; K74C), respectively.
Table 1

B. subtilis strains and plasmids used in this study

| Strain          | Genotype                                      | Source    |
|-----------------|-----------------------------------------------|-----------|
| PY79            | Wild type                                     | (39)      |
| KP775           | ΔspoIIQ::spc                                  | (21)      |
| KP701           | sfpB-lacZ::tet                                | (21)      |
| KP856           | spoIIAH::flag::cat                            | This study|
| KP857           | spoIIAH::flag::Δ::spoIIQ::spc                 | (23)      |
| KP753           | Δ::spoIIQ::spc, thr::cotD-lacZ::lms            | (17)      |
| KP6012          | amyE::spoIII::gfp::cat, Δ::spoIlE::spc        | (33)      |
| KP6111          | amyE::spoIIH124::is31::gfp::cat, Δ::spoIIH::spc| This study|
| AR232           | Δ::spoIIQ::spc, sspB-lacZ::tet                 | This study|
| XJ120           | thr::cotD-lacZ::lms                           | This study|
| XJ140           | Δ::spoIIAH::trpC2                             | This study|
| XJ1412          | spoIIQ::flag::cat                             | This study|
| SCR3            | Δ::spoIIQ::spc, amyE::cat                     | This study|
| SCR4            | Δ::spoIIQ::spc, amyE::p_spoIIQ::QCat          | This study|
| SCR6            | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR9            | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::his::cat| (17)      |
| SCR15           | Δ::spoIIQ::spc, pollig::spoIIQ::J::erm, amyE::P_spoIIQ::QCat| This study|
| SCR18           | Δ::spoIIQ::spc, spb::lacZ::tet, amyE::pollig::QCat| This study|
| SCR21           | Δ::spoIIQ::spc, thr::cotD-lacZ::lms, amyE::P_spoIIQ::QCat| This study|
| SCR43           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR45           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR46           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR47           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR48           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR49           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR50           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR51           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR62           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR63           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR64           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR65           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR66           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR67           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR68           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR69           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR70           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR71           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR72           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR73           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR74           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR75           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR76           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR77           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR78           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR79           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR80           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR81           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR82           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR83           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR84           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR85           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR86           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR87           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR88           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR89           | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR108          | Δ::spoIIQ::spc, sfpB-lacZ::tet, amyE::p_spoIIQ::QCat| This study|
| SCR109          | Δ::spoIIQ::spc, sfpB-lacZ::tet, amyE::p_spoIIQ::QCat| This study|
| SCR110          | Δ::spoIIQ::spc, sfpB-lacZ::tet, amyE::p_spoIIQ::QCat| This study|
| SCR111          | Δ::spoIIQ::spc, sfpB-lacZ::tet, amyE::p_spoIIQ::QCat| This study|
| SCR114          | Δ::spoIIQ::spc, thr::cotD-lacZ::lms, amyE::P_gspB::spoIIQ::QCat| This study|
| SCR115          | Δ::spoIIQ::spc, thr::cotD-lacZ::lms, amyE::P_gspB::spoIIQ::QCat| This study|
| SCR116          | Δ::spoIIQ::spc, thr::cotD-lacZ::lms, amyE::P_gspB::spoIIQ::QCat| This study|
| SCR117          | Δ::spoIIQ::spc, thr::cotD-lacZ::lms, amyE::P_gspB::spoIIQ::QCat| This study|
| SCR138          | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR139          | Δ::spoIIQ::spc, amyE::p_gspB::spoIIQ::QCat    | This study|
| SCR223          | Δ::spoIIQ::spc, thr::cotD-lacZ::lms, amyE::E::cat, bofa::cat::tet, spoIIH::neo| This study|
| SCR224          | Δ::spoIIQ::spc, thr::cotD-lacZ::lms, amyE::E::cat, bofa::cat::tet, spoIIH::neo| This study|
| SCR225          | Δ::spoIIQ::spc, thr::cotD-lacZ::lms, amyE::E::cat, bofa::cat::tet, spoIIH::neo| This study|
| SCR227          | Δ::spoIIQ::spc, thr::cotD-lacZ::lms, amyE::E::cat, bofa::cat::tet, spoIIH::neo| This study|
| SCR228          | Δ::spoIIQ::spc, thr::cotD-lacZ::lms, amyE::E::cat, bofa::cat::tet, spoIIH::neo| This study|
| SCR229          | Δ::spoIIQ::spc, thr::cotD-lacZ::lms, amyE::E::cat, bofa::cat::tet, spoIIH::neo| This study|

pCh687 (gsp-spoIVFA<sup>101–264</sup> FLAG) was constructed as follows: spoIVFA was amplified from PY79 chromosomal DNA using primers SP6 and SP7, digested by BamHI, and then cloned into pDG1662. A FLAG tag was introduced at the C-terminal end of spoIVFA by site-directed mutagenesis using SP8. A BamHI site was further introduced between the 100th and 101st codons by site-directed mutagenesis using primer SP9. A BamHI fragment encoding the C-terminal extracytoplasmic region was cloned into the same site of pGEX4T-3 (Amersham Biosciences).

Sporulation Conditions—Sporulation was induced by resuspension at 37 °C (27), with t<sub>n</sub> being the hours after the onset of
sporulation. For small scale sporulations, the cells were resuspended in 2 ml of the sporulation media and cultured with rotating in test tubes (small scale culture for Fig. 3A). For larger scale sporulations, the cells were resuspended in 15 ml for other Western blotting, β-galactosidase activity assay, and microscopy) or 200 ml (for immunopurification of SpoIIQ-FLAG derivatives in Fig. 2B) and shaken in flasks. Sporulation efficiency was determined as described (28). β-Galactosidase assays were performed as described (29, 30).

Co-immunoprecipitation and Western Blotting—Sporulating cells were harvested and washed with SMM buffer (0.5 mM succrose, 20 mM MgCl₂, and 20 mM maleic acid, pH 6.5), resuspended in the same buffer and treated with 1 mg/ml lysozyme at 37 °C for 10 min. The spheroplasts were harvested by centrifugation and resuspended in ice-cold buffer A (20 mM HEPES-NaOH, 150 mM NaCl, and 1 mM EDTA, pH 7.6) with 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, and 1 µg/ml pepstatin and then treated with 0.5% n-dodecyl β-d-maltoside (Sigma) on ice for 30 min. The insoluble fraction was removed by ultracentrifugation with a Beckman TLA120-2× rotor (40,000 rpm, 30 min), and the supernatant was incubated with anti-FLAG M2 affinity gel (Sigma) overnight at 4 °C with gentle rolling. The affinity gel was washed twice with buffer A containing 0.5% n-dodecyl β-d-maltoside, and the bound proteins were eluted by SDS-loading buffer (without reducing agent) at 42 °C. Proteins in the cell lysate and in the flow-through containing unbound protein were precipitated by 5% trichloroacetic acid for 20 min on ice, washed by acetone, and then solubilized in SDS loading buffer. Proteins were analyzed by SDS-PAGE or Western blotting as described previously (17, 23). 1:5,000 dilutions of anti-SpoIIQ (17), anti-SpoIIIAH (see below), anti-GFP (Roche Applied Science), and anti-FLAG M2 antibodies (Sigma) were used to probe derivatives of SpoIIQ, GFP-SpoIIQ, and SpoIIIAH-FLAG, respectively.

Sucrose Density Gradient Ultracentrifugation—Sucrose density gradient ultracentrifugation was performed essentially as described (31), with the following modifications. Sporulating cell lysates were prepared by the same procedure as described above for co-immunoprecipitation, except that buffer B (20 mM HEPES-NaOH, 300 mM KCl, and 1 mM EDTA, pH 7.6) was used instead of buffer A. The lysates were loaded on top of 5-ml sucrose gradient beds (5–20% sucrose, 20 mM HEPES-NaOH, 300 mM KCl, and 1 mM EDTA, pH 7.6). Proteins were separated by ultracentrifugation in a Beckman MLS50 rotor (4 °C, 43,000 rpm, 16 h) and collected into 17–18 fractions. Purified proteins catalase (226 kDa), adolase (146 kDa, Amersham Biosciences), bovine serum albumin (68 kDa), and lysozyme (14.3 kDa, Sigma) were used as the protein standards.

AMS Modification of Monocysteine Derivatives of SpoIIQ—For AMS treatment, whole cell proteins were solubilized in SDS-loading buffer, containing 1 mM Tris(2-carboxyethyl) phosphate instead of 2 mM dithiothreitol. The pH of the SDS-loading buffer changed by addition of Tris(2-carboxyethyl) phosphate was adjusted to pH 6.8 by adding the proper volume of 0.5 M Tris-HCl (pH 6.8). Samples were treated with 2 mM AMS (4-acetamido-4′-maleimidylstibene-2,2′-disulfonic acid) at 37 °C for 1 h and then analyzed by Western blotting.

In Vivo Proteolysis Assay of SpoIIQ Derivatives—A whole cell trichloroacetic acid precipitation was prepared from 1 ml of culture to which trichloroacetic acid was added (to 5% final concentration). The cells were collected by centrifugation, and the pellet was washed with 0.75 ml of 1 M Tris- HCl (pH 8) and treated with 1 mg/ml lysozyme in 60 µl of buffer C (33 mM Tris-HCl, 40% sucrose, 1 mM EDTA, pH 8). Proteins were then solubilized by SDS-loading buffer with 2 mM dithiothreitol and analyzed by Western blotting.

Purification of SpoIVB, SpoIIQ, and SpoIVFA—BL21(DE3)/ pZR53 (SpoIVB-His₆) was grown in LB ampicillin (100 µg/ml) media, and expression of SpoIVB-His₆ was induced by 1 mM isopropyl-1-thio-β-d-galactopyranoside for 1 h at 30 °C. Cells were washed by buffer D (20 mM Tris-HCl (pH 8.0), 150 mM NaCl) and disrupted by sonication. After removing debris by centrifugation, cell lysate was subjected to nickel affinity column (Sigma) equilibrated in buffer D. The column was washed with buffer D plus 20 mM imidazole and eluted with 300 mM imidazole in the same buffer. Eluted protein was then dialyzed in buffer D, loaded on Hi-Trap Q (Amersham Biosciences) anion-exchange chromatography column previously equilibrated in buffer D. Flow-through fractions that include SpoIVB-His₆ were collected and then loaded onto a Hi-Trap SP (Amersham Biosciences) cation-exchange chromatography column previously equilibrated in buffer D. SpoIVB-His₆ was eluted by a sodium chloride gradient (0–1 M) in buffer D.

GST-SpoIVF and GST-SpoIIQ were expressed in BL21 (DE3). Expression was induced by the addition of 1 mM isopropyl-1-thio-β-d-galactopyranoside for 2–3 h at 37 °C. Cells were washed with phosphate-buffered saline (PBS), suspended in PBS/1 mM dithiothreitol/1 mM EDTA, and disrupted by sonication. Cell lysate was loaded on a glutathione-Sepharose column (Amersham Biosciences) previously equilibrated in PBS, washed with PBS, and eluted with 50 mM Tris-HCl (pH 8.0) containing 10 mM glutathione. GST-SpoIVFA₁⁰—₂⁶₄-FLAG was dialyzed by buffer D and further purified by using a Sephacryl S-200 column (Amersham Biosciences). All proteins were dialyzed in buffer D, and concentration was determined by a Bradford protein assay (Sigma).

In Vitro Protease Assay and Amino Acid Sequence Analysis—Purified substrates (240 µg/µl) were incubated in buffer D at 37 °C in 0–4 h in the presence or absence of 30 ng/µl purified SpoIVB-His₆. The reaction was stopped with SDS-loading buffer, and the products were analyzed by SDS-PAGE and Coomassie staining. Bands were excised from polyvinylidene difluoride membrane and subjected to N-terminal sequencing.

Microscopy and Image Analysis—For GFP visualization, live cells were stained with 4′,6′-diamidino-2-phenylindole (0.2 µg/ml, Molecular Probes) and FM4-64 (5 µg/ml, Molecular Probes) as described previously (32). Images were collected with an Applied Precision Spectris microscope with a QLM laser module (described in Ref. 33). Photobleaching was performed and quantified as described (22), using a 0.02- to 0.05-s pulse of a 488 nm argon laser at 50% power. Subsequent GFP images were collected at 30-s intervals for 5 min for GFP-SpoIIQ and V72Y or as quickly as possible for smoothly localized GFP-SpoIIQV72Y. Exposure times were limited to 1.5–2.5 s. These experiments were quantified as previously described (22).
Membrane Fusion Modulates SpoIIQ Dynamics and Proteolysis

**FIGURE 1.** *B. subtilis* engulfment, SpoIIQ proteolysis, and $\alpha^0$ and $\alpha^K$ activation. A, after septation, the larger mother cell engulfs the smaller forespore, which is ultimately completely enclosed in the mother cell cytoplasm. GFP-SpoIIQ (dark gray) assembles foci at the septal midpoint and migrates around the forespore with the engulfing membrane, assembling a helical or hexagonal structure in the forespore (19). After engulfment, SpoIIQ is cleaved to release the N-terminal GFP fragment into the forespore cytoplasm (17, 19). The forespore the engulfing membrane, assembling a helical or hexagonal structure in the forespore (19). After engulfment, SpoIIQ is required to process SpoIVB (17). SpoIVB is also required to process SpoIIQ (17).

B. *B. subtilis* SpoIVB cleaves SpoIIQ. GFP-SpoIIQ, which is present in the cytoplasm, is cleaved to release the extracellular domain (corresponding to amino acids 104–123 of SpoIIQ) into the extracellular space. The first initiating proteolysis event ($\alpha^0$) is coupled to engulfment by a BofA-independent mechanism (17). BofA-independent mechanism (17). The interacting proteins SpoIIQ (Q) and SpoIIAH (AH) are required for activation of $\alpha^0$ and recruit the BofA-SpoVFA-SpoVFB complex necessary for activation of $\alpha^0$ to the outer forespore membrane (OFM) (17, 24). Activation of $\alpha^0$ is governed by two checkpoints: the forespore checkpoint (i) in which $\alpha^0$ activation allows the production of high levels of SpoIVB (11), which cleaves SpoIVFA to release SpoIVB from inhibition by BofA, and the engulfment checkpoint (ii) in which $\alpha^0$ activity is coupled to engulfment by a BofA-independent mechanism (17). SpoIVB is also required to process SpoIIQ (17). C, SpoIIQ traverses the forespore membrane (FM), with a cytoplasmic N terminus that is the site of the GFP fusion. The extracellular C-terminal domain was used as the antigen for polyclonal antibodies (anti-IIQ) (17) while His6 and FLAG tags were fused to the extreme C terminus. Three proteolysis events are indicated by roman numerals (I–III). The first initiating proteolysis event (I) requires SpoIVB serine protease (lightning bolt), and allows a subsequent cleavage within the membrane or in the cytoplasm (II). There might also be a third cleavage event (III) near the C terminus of the protein that releases the His6 and FLAG tags. D, models for the role of SpoIIQ in engulfment-dependent gene expression. D, the "pre-proteolytic activation model," in which full-length SpoIIQ is required for $\alpha^0$ and $\alpha^K$ activity; E, "post-proteolytic activation model," in which SpoIIQ proteolysis mediates intracellular signal transduction.

Briefly, the images were corrected for photobleaching during image collection, and the fluorescence intensity of the bleached and unbleached regions were quantified throughout the experiment, and corrected for bleaching during image acquisition. The small size of the forespore results in a small and variably sized pool of unbleached GFP-SpoIIQ from which recovery can occur. We therefore calculated the theoretical equilibration point between the bleached and unbleached regions, which is represented as a dashed line.

**RESULTS**

Identification of in Vivo Cleavage Site of SpoIIQ—Previous results demonstrated that both N- and C-terminal proteolytic products of SpoIIQ, GFP-SpoIIQ, and SpoIIQ-His$_6$ were soluble, suggesting that the protein was cleaved on both sides of the membrane (Fig. 1C) (17). The ability to completely block SpoIIQ proteolysis with spoIVB mutations suggested that SpoIIQ proteolysis is initiated by the secreted SpoIVB protease, which releases the extracellular domain from the membrane, and allows a subsequent cytoplasmic (or perhaps intramembrane) proteolysis that releases the N-terminal GFP tag into the cytoplasm (Fig. 1C) (17). There might also be a third proteolysis event near the C terminus of SpoIIQ (Fig. 1C), based on our analysis of SpoIIQ-His$_6$. This protein shows two proteolytic products (Fig. 2A) in immunoblot experiments with anti-SpoIIQ antibodies, one slightly larger than that of native SpoIIQ (which reacts with His$_6$-specific antibodies) and one identical in size to that of native SpoIIQ (and that does not react with His$_6$-specific antibodies).

To further characterize the SpoIVB-dependent proteolysis event that appears to initiate SpoIIQ degradation we used two independent methods to identify the site at which SpoIIQ was cleaved in intact cells.

First, we immunoprecipitated SpoIIQ-FLAG from whole cell lysates and extracted a band of ~25 kDa that appeared only when SpoIIQ-FLAG was expressed (Fig. 2B, lane 2). The major signal from the amino acid sequencing analysis was GKSME (corresponding to amino acids 73–78 of SpoIIQ), suggesting that extracellular cleavage occurs between Val-72 and Gly-73. Second, we constructed a series of SpoIIQ derivatives with individual cysteines introduced between codons 62 and 74 and used AMS modification (35) to determine if the cysteine residue was present in the C-terminal degradation product, which confirmed that in intact cells proteolysis occurred between Val-72 and Gly-73 (supplemental Fig. S1).

**Purified SpoIVB Cleaves SpoIIQ**—To determine if SpoIVB (a serine protease) directly cleaves SpoIIQ, we tested if purified SpoIVB cleaved purified GST-SpoIIQ in vitro, using GST-SpoIVFA (a known SpoIVB substrate), GST and $\alpha$- and $\beta$-casein as control proteins. SpoIVB was able to cleave GST-SpoIIQ and GST-SpoIVFA with similar kinetics (Fig. 2C),
Membrane Fusion Modulates SpoIIQ Dynamics and Proteolysis

FIGURE 2. Identification of SpoIVB cleavage sites in SpoIIQ and SpoIVFA. A, in vivo proteolysis of SpoIIQ (PY79) and SpoIIQ-His<sub>6</sub> (SCB9) analyzed by Western blot using anti-SpoIIQ polyclonal antiserum at various times of sporulation. Full-length SpoIIQ (IIQ-FL) migrates at ~37 kDa, the C-terminal product (C-term) at ~26 kDa. B, SpoIIQ-FLAG (lane 2, X4142) and the negative control strain PY79 (lane 1) were immunoprecipitated with anti-FLAG antibodies, and visualized by Coomassie staining. The bands indicated by C-term in lane 2 and lane 1 were extracted and subjected to N-terminal amino acid sequencing. GST-SpoIIQ was cleaved once to yield a GST-con- 

whereas GST was stable during the incubation (data not shown). The degradation products of GST-SpoIIQ and GST-SpoIVFA were excised and subjected to N-terminal amino acid sequencing. GST-SpoIIQ was cleaved once to yield a GST-containing product (G1) and a product with an N-terminal sequence starting at Gly-73 of SpoIIQ (IIQ1). Thus, purified SpoIVB cleaves SpoIIQ between Val-72 and Gly-73, the same position at which SpoIIQ is cut in intact cells. SpoIVB cleaved GST-SpoIVFA and itself in three locations (Fig. 2E) (36). A comparison of the SpoIVB cleavage sites of these three proteins and α- and β-casein demonstrated that in each case SpoIVB cut SpoIIQ was cleaved by t<sub>5</sub> (Fig. 3A, lanes 1 and 28). Of the 29 mutants tested (including three Cys substitutions, supplemental Fig. S1), 14 were cleavage-defective, and 15 were permissive. At amino acid 72 only V72A and V72C allowed proteolysis (Fig. 3A, lane 27), although introduction of negatively charged amino acids (Glu or Asp) or proline inhibited proteolysis. Alignment of the N-terminal regions of SpoIIQ from various Bacillus species (Fig. 3C) demonstrated that each had

after either alanine (in the case of SpoIVFA, caseins, and one site in SpoIVB) or valine (in the case of SpoIIQ and two sites in SpoIVB), with little other primary sequence similarity in the surrounding region. A similar analysis by another group also identified these three sites in SpoIVFA (15). Using mass spectrometry analysis, they also observed cleavage at an EVGK motif within SpoIVFA that is identical to the site of proteolysis in SpoIIQ (15). Cleavage at this site depended on cleavage at the most C-terminal cleavage site. This explains why our N-terminal sequencing analysis failed to detect cleavage at EVGK, because we sequenced only the larger C-terminal products, which will have the same N terminus with or without cleavage at EVGK. The 8-amino acid internal product of cleavage at these sites is too small to be detected by SDS-PAGE.

Isolation of Cleavage-defective SpoIIQ Proteins—The above results demonstrate that SpoIIQ is directly cleaved by the SpoIVB serine protease that is essential for σ<sup>II</sup> activation. To determine if SpoIIQ proteolysis was essential for sporulation, we used site-directed mutagenesis to randomize codons 72, 73, and 74 of wild-type SpoIIQ (“Experimental Procedures”), isolating 26 different amino acid substitutions at these sites. The mutations were introduced into B. subtilis and screened for defects in SpoIIQ proteolysis using small scale sporulations (in test tubes) and anti-SpoIIQ Western blots from extracts prepared 5 h after the initiation of sporulation (t<sub>5</sub>, Fig. 3A). Under these conditions, sporulation is slowed, likely due to decreased aeration, but wild-type

JANUARY 26, 2007 • VOLUME 282 • NUMBER 4 JOURNAL OF BIOLOGICAL CHEMISTRY 2581
Membrane Fusion Modulates SpoIIQ Dynamics and Proteolysis

FIGURE 3. Protease sensitivity and αG and αK activity of SpoIIQ cleavage site-mutants. A, proteolysis in small-scale cultures. Sporulation was induced by resuspension in a 2-ml culture in test tubes, and samples were prepared after 5 h at 37 °C for Western blot analysis with anti-SpoIIQ. Amino acids introduced at Val-72 (lanes 2–11), Gly-73 (lanes 12–18), and Lys-74 (lanes 19–27) are indicated. B, time course of proteolysis in large scale cultures. IIQ-FL and C-term indicate full-length SpoIIQ and C-terminal cleavage products. C, alignment of predicted cleavage sites of SpoIIQ from various Bacillus sp. The arrowhead indicates cleavage site of B. subtilis SpoIIQ. D–F, affect of various spoIIQ mutations on αG and αK activity. All strains contained the lacZ fusion indicated in each panel, and the spoIIQ mutation indicated by the following symbols (circles, spoIIQG; squares, ΔspoIIQ; triangles, wt; inverted triangles, V72C; solid diamonds, V72M; open diamonds, G73V; solid circles, G73E). In addition, all strains with a spoIIQ derivative at amyE also contained a spoIIQ null mutation. D, to assay αG activity, strains K701, AR232, SCB18, SCB108, SCB109, SCB110, and SCB111 were used. E, for αK activity, XJ220, KP953, SCB21, SCB114, SCB115, SCB116, and SCB117 were used. F, the cleavage site mutants also supported αK activity in strains lacking spoIIQ and bofA. Strains contained the indicated spoIIQ mutations plus bofA and spoIIQ (SCB223, SCB224, SCB225, SCB227, SCB228, and SCB229).

To distinguish between these models, we compared the levels of αG and αK activity (using sspB-lacZ and cotD-lacZ, respectively) in strains carrying mutations that block (V72M and G73E) or allow (V72C and G73V) SpoIIQ proteolysis. These mutants showed αG and αK activities nearly identical to wild type (Fig. 3, D and E). Thus, the proteolysis-defective mutants have no effect on αG or αK activity, suggesting that SpoIIQ proteolysis is not essential for engulfment-dependent gene expression, in keeping with the pre-proteolytic model.

The activation of αK is governed by two checkpoints, the forespore checkpoint (11) and a cellular signal transduction via an RIP-like mechanism (post-proteolytic model in Fig. 1E). However, full-length SpoIIQ also acts during engulfment to recruit mother cell membrane proteins required for αG and αK activation to the septum (17, 23, 24), raising the possibility that an event that occurs before proteolysis might mediate extracellular signal transduction via an RIP-like mechanism (post-proteolytic model in Fig. 1D).

valine at the predicted SpoIVB cleavage site and permissive substitutions at positions corresponding to Gly-73 and Lys-74 of B. subtilis SpoIIQ (aside from Geobacter and Oceanobacillus iheyensis, which had a non-permissive substitution at position 74). Thus, valine appears to be conserved at the cleavage site of SpoIIQ proteins that are sufficiently closely related to allow a reliable alignment of their N-terminal domain.

We more precisely followed degradation of three cleavage-defective mutants and two permissive mutants using larger scale sporulations (Fig. 3B). Each of the cleavage-defective mutants showed very slow and inefficient degradation, with little accumulation of the C-terminal product and no notable decrease in the levels of full-length SpoIIQ even after 5 h of sporulation. In contrast the permissive mutants showed normal or slightly slower (V72C) degradation (Fig. 3B), accumulating a stable C-terminal breakdown product. To determine if these proteins were equally resistant or sensitive to in vitro proteolysis by purified SpoIVB, we introduced the cleavage-defective substitution V72M and the permissive substitution V72A into GST-SpoIIQ. As expected, V72M was resistant to SpoIVB cleavage at the usual cleavage site, although it generated a larger proteolysis product that might correspond to cleavage at a more C-terminal SpoIVB cleavage (G2 in Fig. 2C). We could find no evidence that SpoIVB cleaved V72M at this site in vivo, because no intermediate size product was observed in whole cell extracts (Fig. 3B). Interestingly, V72A, which is permissive for proteolysis in intact cells, was degraded in vitro more slowly than wild-type SpoIIQ (Fig. 2C).
bypassed by a bofA mutation, which allows $\sigma^K$ activation in the absence of $\sigma^G$ (37), but the bofA mutation does not bypass either engulfment or the requirement for SpoIIG and SpoIIAH for $\sigma^K$ activity (17). We tested whether cleavage-defective SpoIIQ supported $\sigma^K$ activation in a bofA mutant lacking $\sigma^G$, by introducing the cleavage-defective or -permissive mutants into a cell that has null mutations in spoIIIG (encoding $\sigma^G$), bofA and spoIIQ. Again, both cleavage-defective and cleavage-permissive mutations supported $\sigma^K$ activation in these assays, with a slight reduction in activity seen in both classes of mutations at late times (Fig. 3F).

Thus, SpoIVB-mediated proteolysis of SpoIIQ is dispensable for both $\sigma^G$ and $\sigma^K$ activity, as well as for the production of heat-resistant spores (Table 2). These results suggest that either SpoIIQ proteolysis is normally dispensable for sporulation or that cytoplasmic proteolysis does not depend on SpoIVB-mediated extracellular proteolysis.

**Cytoplasmic Cleavage of SpoIIQ Depends on Extracytoplasmic Cleavage** —The absence of smaller breakdown products in SpoIIQ proteins resistant to SpoIVB-mediated extracellular proteolysis (Fig. 3) and in the absence of SpoIVB (17) suggests that, in intact cells, cytoplasmic proteolysis occurs after extracellular proteolysis. To further test this hypothesis, we introduced the cleavage-resistant mutations V72Y and V72E into GFP-SpoIIQ and tested whether the cytoplasmic cleavage occurred to release the N-terminal GFP tag into the cytoplasm. Western blot analysis demonstrated that GFP-SpoIIQ was partially stabilized by these mutations, with a decreased amount of the N-terminal GFP-containing breakdown product relative to wild type at $t_5$ (Fig. 4A). Prior studies have demonstrated that GFP-SpoIIQ is more permissive for proteolysis than native SpoIIQ. Unlike the wild-type protein, GFP-SpoIIQ is not completely stabilized by the absence of either SpoIVB or SpoIIAH (17). This proteolysis might occur during sample preparation, because fluorescence microscopy demonstrated that, similar to GFP-SpoIIQ in the spoIVB and spoIIAH mutants (17), the proteolysis-resistant mutant V72Y remained membrane-bound after engulfment, with little cytoplasmic GFP fluorescence (Fig. 4B). Together

| Strain | Genotype | Cleavage | Spore titer |
|--------|----------|----------|-------------|
| PY79   | spoIIQ+  | +        | 2.7 x 10^8  |
| SCB3   | ΔspoIIQ::spc, amyE:cat | +        | 0 x 10^8    |
| SCB4   | ΔspoIIQ::spc, amyE::spoIIQcat | +        | 2.5 x 10^8  |
| SCB50  | ΔspoIIQ::spc, amyE::spoIIQV72ICat | +        | 2.6 x 10^8  |
| SCB71  | ΔspoIIQ::spc, amyE::spoIIQV72ICat | -        | 2.0 x 10^8  |
| SCB72  | ΔspoIIQ::spc, amyE::spoIIQG73ICat | -        | 1.7 x 10^8  |
| SCB73  | ΔspoIIQ::spc, amyE::spoIIQI74ICat | -        | 2.0 x 10^8  |
| SCB83  | ΔspoIIQ::spc, amyE::spoIIQI74ICat | -        | 1.8 x 10^8  |
| SCB80  | ΔspoIIQ::spc, amyE::spoIIQI74ICat | -        | 1.9 x 10^8  |
Membrane Fusion Modulates SpoIIQ Dynamics and Proteolysis

with the ability of mutations at the site of extracellular cleavage to completely stabilize native SpoIIQ (with no slightly smaller breakdown products, Fig. 3), these results suggest that cytoplasmic cleavage of SpoIIQ normally depends on its extracellular cleavage by SpoIVB (Fig. 1C).

This mechanism of SpoIIQ proteolysis shows similarity to that of RIP, in which degradation of a signal transduction protein is typically initiated by an extracellular cleavage that allows a second intracellular or intramembrane cleavage by a second protease. However, despite the observation that SpoIIQ proteolysis is governed by the same morphological checkpoint (engulfment) and protease (SpoIVB) as late mother cell gene expression, we found no evidence that it participates in intracellular signal transduction.

Release of SpoIIQ from an Immobile Complex after Engagement—We reasoned that the completion of engulfment might mediate a rearrangement in the complex between SpoIIQ and its mother cell ligand SpoIIIAH or its unidentified tether (19, 23) and that this rearrangement might allow both SpoIIQ proteolysis and signal transduction. If this is the case, then SpoIIQ might show different diffusion kinetics before and after the membrane fusion event that is the final step of engulfment. Indeed, a FRAP analysis of the diffusion kinetics of wild-type GFP-SpoIIQ during and after engulfment demonstrated that SpoIIQ is relatively immobile during engulfment with somewhat increased mobility after engulfment (22). However, wild-type SpoIIQ is degraded after engulfment, so this could reflect loss of the extracellular domain rather than release from a complex. We therefore performed a FRAP analysis of the protease-resistant mutant V72Y. During engulfment, V72Y showed the same restricted mobility as wild-type SpoIIQ (supplemental Fig. S2) (22), with equilibration between the bleached and unbleached regions requiring at least 200 s (supplemental Fig. S2) (22). After engulfment, V72Y showed both the punctate localization seen in wild type before proteolysis, and a smooth localization pattern not observed in wild type (Fig. 4B), unless it is expressed in the absence of SpoIVB (17). These two patterns each had distinct FRAP results (Fig. 4C): sporangia with punctate localization showed low mobility (equilibrium times or \( t_{eq} \approx 240 \) s), whereas those with smooth localization showed a high mobility (\( t_{eq} \approx 10 \) s) similar that of forespore-expressed MalFTM1–2-GFP (\( t_{eq} \approx 6 \) s) (22). We obtained essentially the same results with another cleavage-defective SpoIIQ protein (V72E), and wild-type SpoIIQ in the proteolysis-defective spoIVB strain (supplemental Fig. S2).

Thus after membrane fusion, protease-resistant SpoIIQ is released from a punctate or helical structure in which it is essentially immobile, attaining a rapid diffusion rate similar to a non-localized protein. This event could allow both RIP and intracellular signal transduction necessary for engulfment-dependent gene expression in the forespore and the mother cell.

Reduced Interaction between the Extracellular Proteolysis Product of SpoIIQ and SpoIIIAH—We were interested in determining if the proteolytic products of SpoIIQ interacted with its mother cell ligand SpoIIIAH. We therefore used non-denaturing sucrose density gradient analysis (31) to compare the apparent molecular weight of full-length SpoIIQ, its C-terminal degradation product, and SpoIIIAH. Full-length SpoIIQ and SpoIIIAH were present in the same fractions (Fig. 5A, with apparent molecular masses of ~100 kDa, significantly higher than their predicted molecular masses of 31 kDa and 24 kDa, respectively. The high molecular weight was likely a consequence of the interaction between SpoIIQ and SpoIIIAH, because both proteins had a reduced apparent molecular mass in strains lacking the other protein (~40 kDa). The level of SpoIIIAH was reduced in the absence of SpoIIQ (Fig. 5A, supplemental Fig. S3D), suggesting that SpoIIQ protects SpoIIIAH from proteolysis.

Samples harvested after SpoIIQ proteolysis commenced demonstrated that, although the differences in molecular mass between full-length SpoIIQ and its C-terminal product is only ~8 kDa (31 kDa versus 23 kDa), these two proteins showed strikingly different apparent sizes, with full-length SpoIIQ behaving as a ~100 kDa protein, and the C-terminal product as a ~30 kDa protein (Fig. 5B). This suggests a reduced interaction between the C-terminal fragment of SpoIIQ and SpoIIIAH. We confirmed this hypothesis by immunoprecipitating SpoIIIAH–FLAG and probing the eluate with anti-SpoIIQ antibody. While full-length SpoIIQ was efficiently co-precipitated with SpoIIIAH–FLAG, the C-terminal fragment of SpoIIQ was not precipitated (Fig. 5C). Together these results indicate that there is any interaction between the C-terminal degradation product of SpoIIQ and SpoIIIAH, the affinity is too low to be detected by either sucrose density gradient analysis or co-immunoprecipitation.

SpoIVB-mediated SpoIIQ proteolysis occurs after V72, leaving the N-terminal transmembrane segment and almost 30
Membrane Fusion Modulates SpoIIQ Dynamics and Proteolysis

amino acids outside the cell that might interact with SpoIIIAH. In an attempt to determine if this region interacted with SpoIIIAH, we expressed the N-terminal fragment of SpoIIQ1–72 and used immunofluorescence and Western blotting to determine if it was able to recruit SpoIIIAH to the sporulation septum and protect it from proteolysis. SpoIIIAH localization and stability was reduced in the SpoIIQ1–72 strain. This might be due to the rapid degradation of the N-terminal fragment, because GFP fusions to the first 60 or 72 amino acids of SpoIIQ were quickly degraded to soluble GFP (data not shown), as is the N-terminal cleavage product of full-length GFP-SpoIIQ (which fails to accumulate). This suggests that the N-terminal product of SpoIVB cleavage is too unstable to sustain an interaction with SpoIIIAH. Thus, SpoIIQ appears to make a high affinity interaction with SpoIIIAH only after proteolysis, raising the possibility that premature degradation of SpoIIQ might compromise its ability to recruit SpoIIIAH and other proteins to the sporulation septum.

SpoIIQ Proteolysis Occurs in a Membrane Fusion-dependent Manner—Although SpoIVB is synthesized during engulfment (18), three lines of evidence indicate that SpoIVB-mediated proteolysis of SpoIIQ occurs only after engulfment. First, cell biological studies of GFP-SpoIIQ degradation demonstrate that the N-terminal GFP moiety is released from the membrane only in sporangia that have completed membrane fusion, demonstrating that cytoplasmic proteolysis occurs only after fusion (19). Second, Western blot analysis of native SpoIIQ (with anti-SpoIIQ antiserum) demonstrates that low levels of SpoIIQ proteolysis is first observed at 2.5 h of sporulation (Fig. 5C), consistent with observations that membrane fusion is first completed in a few sporangia just before 2 h of sporulation (at 105 min (32)). Finally, proteolysis of native SpoIIQ does not occur in spoIID or spoIIIP mutants, which block engulfment prior to the onset of membrane migration (17).

We were interested in determining if SpoIIQ proteolysis was regulated by membrane migration or by the membrane fusion event that is the final step of engulfment. To address this question, we made use of a spoIIIIE mutant that completes membrane migration but not membrane fusion (and which translocates DNA (33)). The membrane fusion-defective mutant abolished degradation of native SpoIIQ (Fig. 6B) and accumulated full-length SpoIIQ. These results indicate that SpoIVB-mediated degradation of SpoIIQ depends on the final step of engulfment, membrane fusion, in keeping with prior cell biological studies.

DISCUSSION

Our results demonstrate that SpoIIQ is directly cleaved by the SpoIVB protease that is also required for activation of the late mother cell transcription factor σK. Interestingly, both σK activation and SpoIIQ proteolysis depend on the phagocytosis-like process of engulfment (17, 38). We here provide evidence that SpoIIQ proteolysis more specifically depends on the final step of engulfment, membrane fusion, which releases the forespore into the mother cell cytoplasm. Although SpoIIQ proteolysis is not essential for sporulation, it in some ways provides a better model for SpoIVB-mediated proteolysis than the other identified SpoIVB substrate, SpoIVFA. Specifically, unlike SpoIVFA, SpoIIQ proteolysis releases stable proteolysis products that can readily be detected by both immunoblot analysis and cell biological methods. SpoIIQ thereby provides a tractable system to investigate the mechanism by which SpoIVB activity is governed by the morphological checkpoint of engulfment.

The Checkpoint for SpoIIQ Proteolysis Might Retain the Active Signal Transduction Complex at the Septum—It remains unclear why SpoIIQ is subject to engulfment-dependent proteolysis, because we have failed to identify any phenotypic consequence of blocking SpoIIQ proteolysis. However, our results allow us to propose a reason for why it is important to delay SpoIIQ proteolysis until after the completion of membrane fusion. The interaction between SpoIIQ and SpoIIIAH is required to retain SpoIIIAH and SpoIVFB in the outer forespore membrane (17, 23, 24), where they are involved in intracellular signal transduction cascades that result in activation of late forespore and mother cell transcription factors. We have been unable to detect an interaction between the SpoIIQ proteolysis products and SpoIIIAH in living cells. Thus, if SpoIIQ were degraded prior to membrane fusion the decreased affinity of the interaction between SpoIIQ and SpoIIIAH might result in the release of SpoIIIAH and SpoIVFB from the outer forespore membrane (Fig. 6B), thereby compromising intracellular signal transduction. Thus, using membrane fusion as a checkpoint for SpoIIQ proteolysis might serve to maintain the interaction between SpoIIQ and SpoIIIAH and SpoIVFB until after engulfment, ensuring that proteins required for σK and σG activation localize exclusively to the outer forespore membrane where cell-to-cell communication occurs (Fig. 6C).

A Membrane Fusion-dependent Reorganization in the SpoIIQ-SpoIIIAH Complex—Our studies also suggest a membrane fusion-dependent rearrangement in the complex be-
Membrane Fusion Modulates SpoIIQ Dynamics and Proteolysis

tween SpoIIQ, SpoIIIAH, and other proteins involved in late forespore and mother cell gene expression. Specifically, our FRAP studies demonstrate that, although SpoIIQ is essentially immobile during engulfment (22), after membrane fusion it diffuses through the forespore membrane at rates nearly identical to a non-localized MalF-GFP protein (Fig. 4). This result is most easily interpreted as reflecting a remodeling of the interaction between SpoIIQ and its binding partner, SpoIIIAH (or another unidentified SpoIIQ-interacting protein), an event that could easily initiate intracellular signal transduction. It is therefore tempting to speculate that remodeling of the SpoIIQ complex provides a signal both for $\sigma^G$ and $\sigma^K$ activation and for SpoIIQ proteolysis, because this would explain both the coordinate regulation of these events by membrane fusion and the dispensable nature of SpoIIQ proteolysis.

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