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Analysis of the Interaction between the Transcription Factor σ^G and the Anti-Sigma Factor SpoIIAB of *Bacillus subtilis*

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The activation of σ^G, a transcription factor, in *Bacillus subtilis* is coupled to the completion of engulfment during sporulation. SpoIIAB, an anti-sigma factor involved in regulation of σ^F, is also shown to form a complex with σ^G in vitro. SpoIIAA, the corresponding anti-anti-sigma factor, can disrupt the SpoIIAB:σ^G complex, releasing free σ^G. The data suggest the existence of an as-yet-unknown mechanism to keep σ^G inactive prior to engulfment.

Starvation induces the gram-positive bacterium *Bacillus subtilis* to initiate a simple, two-cell developmental process that results in the formation of dormant spores. Early in sporulation, the developing cell divides asymmetrically to produce a smaller compartment, the prespore, which becomes the spore, and a larger compartment, the mother cell, which participates in the maturation of the spore and finally lyses to release it. The different developmental fates of the two cells are governed by the sequential activation of four sporulation-specific transcription factors, beginning with σ^F in the prespore and then σ^E in the mother cell followed by σ^G and σ^K in the prespore and mother cell, respectively. To ensure the correct sequence of morphological events, the activation of each sigma factor is coupled to morphogenesis and/or to events occurring in the opposite cell (reviewed in references 11 and 22).

The late prespore-specific σ factor, σ^G, is regulated at at least three levels. First, its gene (*sigG* or *spoIIIG*) is transcribed from a promoter recognized by the first prespore-specific sigma factor, σ^F (and later by σ^G itself), thus restricting its localization to the prespore compartment (24). Second, unlike other σ^F-dependent genes, *sigG* is not transcribed in the presence of mutations in the *spoIIG* gene (20), which encodes the first mother cell-specific sigma factor, σ^E. Therefore, *sigG* transcription is also dependent on an as-yet-unidentified signal transduction pathway of which at least one component is expressed in the mother cell. The third regulatory mechanism exerted over σ^G acts at the level of protein activity. The *sigG* gene begins to be transcribed approximately 120 min after the initiation of sporulation. However, σ^G-dependent gene expression does not begin until 30 min later (20). Mutations in several different genes, including *spoIIB*, *spoIID*, *spoIIM*, *spoIIIA*, and *spoIIIJ*, prevent transcription of σ^G-dependent genes without affecting σ^G synthesis, implying that their products play a role in σ^G activation (1, 8, 9, 12, 15, 20, 21, 23). Three of the proteins, SpoIIB, SpoIID, and SpoIIM, are required for prespore engulfment, suggesting a link between activation of σ^G and the completion of engulfment (1, 21, 23). Little is known about how σ^G is held inactive prior to engulfment, but it has been suggested to involve the anti-sigma factor SpoIIAB (9). SpoIIAB is one of the proteins that regulate σ^F activation; it binds to σ^F and thereby prevents it from interacting with core RNA polymerase (2, 7, 16). In turn, SpoIIAB is antagonized by the anti-anti-sigma factor SpoIIAA. SpoIIAB and SpoIIAA can interact in two different ways. In the presence of ADP, the two proteins form a complex, resulting in the release of active σ^F (2, 5). However, SpoIIAB is also a protein kinase which (in the presence of ATP) phosphorylates SpoIIAA on a specific serine residue, rendering the product (SpoIIAA-P) unable to bind to SpoIIAB or to react with SpoIIAB:σ^F complexes (5, 6, 13, 16, 18). There is some evidence that SpoIIAB also regulates σ^G activity. Constitutive expression of SpoIIAB was found to repress σ^G activity in cells where σ^G was expressed from a σ^F-independent promoter (10). Biochemical cross-linking experiments using radiolabeled crude extracts from *Escherichia coli* strains overexpressing SpoIIAB and σ^G also indicated an interaction between the two proteins (9). Furthermore, σ^G activity is reduced drastically in a *spoIIIA* mutant that fails to degrade SpoIIAB in the prespore (9). The requirement for *spoIIIA* is bypassed partially by a σ^G mutant that is impaired in its interaction with SpoIIAB (9).

In this report, we show that purified σ^G and SpoIIAB proteins form a nucleotide-dependent complex, although the interaction is much weaker than that of σ^F and SpoIIAB. Furthermore, we demonstrate that purified SpoIIAA efficiently disrupts the SpoIIAB:σ^G complex, thereby releasing σ^G. Taken together, the data suggest that SpoIIAB interacts with σ^G in the same way as with σ^F and that there may be another mechanism to keep σ^G inactive at a time when σ^F is active.

Purified σ^G and SpoIIAB form a complex in the presence of nucleotide. σ^G was purified using the IMPACT T7 system (New England BioLabs). The *sigG* gene of *B. subtilis* was amplified by PCR, and the product was cloned into the expression vector pTYB1, thereby fusing the *sigG* gene to an intein-chitin binding domain under the control of the IPTG-inducible T7 promoter. The fusion protein was overproduced in *E. coli* C41(DE3) (17) and purified on a chitin column as described in the New England BioLabs protocol. σ^G was purified further on

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a Superdex 75 gel filtration column equilibrated in 50 mM Tris-HCl (pH 8)–300 mM NaCl–1 mM dithiothreitol(DTT)–1 mM EDTA to remove copurified proteins. Results with the purified protein are shown in Fig. 1A. Using the IMPACT T7 system followed by a Superdex 75 gel filtration column, we also purified σ^F as a control (Fig. 1A) (I. Lucet, unpublished data). To examine whether σ^G forms a complex with SpoIIAB as σ^F does, we incubated increasing amounts of σ^G or σ^F (1, 2, or 4 μM) with purified SpoIIAB (2 μM) in a 30-μl mixture containing 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 3 mM $MgCl_2$, and either ATP or ADP (1 mM) on ice for 30 min. The mixtures were subjected to 10% nondenaturing polyacrylamide gel electrophoresis (PAGE) with the appropriate nucleotide in the running buffer. The proteins were visualized by Coomassie blue staining. As shown in Fig. 1B, in the presence of ATP the SpoIIAB band decreased in intensity with increasing amounts of sigma factor and, in parallel, SpoIIAB(ATP):σ^F (lanes 2 to 4) and SpoIIAB(ATP):σ^G (lanes 5 to 7) complexes appeared that ran with a mobility intermediate between that of SpoIIAB and σ^F or σ^G. In contrast to the SpoIIAB(ATP):σ^F complexes, SpoIIAB(ATP):σ^G complexes ran in a smear rather than in a sharp band, suggesting that the complexes dissociated to some extent during the electrophoresis. In the presence of ADP, only a weak band corresponding to SpoIIAB(ADP):σ^G complexes was detected, suggesting that the complexes are unstable and dissociate rapidly during electrophoresis (Fig. 1C, lanes 5 to 7). In contrast, SpoIIAB and σ^F did form complexes in the presence of ADP (Fig. 1C, lanes 2 to 4), although the bands had a lower intensity than those formed in the presence of ATP. It was shown previously that SpoIIAB(ADP):σ^F complexes are less stable than those formed with ATP (14). A second band running just above the SpoIIAB band was also seen (open arrow head). This could be a monomer of SpoIIAB interacting with σ^F instead of a dimer. No complexes were formed in the absence of any nucleotide (data not shown), showing that nucleotide is required for this interaction just as it is for the interaction of σ^F with SpoIIAB (6). SpoIIAB was also incubated with purified σ^B (1, 2, or 4 μM), a closely related sigma factor of *B. subtilis*, which is known to be regulated by an anti-sigma factor that is similar to SpoIIAB (3). As shown in Fig. 1D (lanes 2 to 4), the two proteins did not form a detectable complex, demonstrating that the binding of SpoIIAB to σ^F and σ^G is specific. These results strongly suggested that in vitro, SpoIIAB binds to σ^G in the same nucleotide-dependent manner as that with which it binds to σ^F, although it appeared that the SpoIIAB:σ^G complexes are less stable on nondenaturing PAGE than SpoIIAB:σ^F complexes are. The data were also in agreement with experiments using chemical cross-linking of crude extracts of *E. coli* designed to express SpoIIAB and σ^G (9) that showed nucleotide-dependent binding between SpoIIAB and σ^G. In addition, it is apparent from the recently solved crystal structure of a SpoIIAB$_2$:σ^F complex that the region of σ^F that interacts is highly conserved in σ^G (4).

One possible mechanism to ensure the inhibition of σ^G under conditions in which σ^F is fully active would be for SpoIIAB to have different affinities for σ^F and σ^G so that when σ^G is produced SpoIIAB binds to it in preference to σ^F. However, the results described above suggested that the interaction

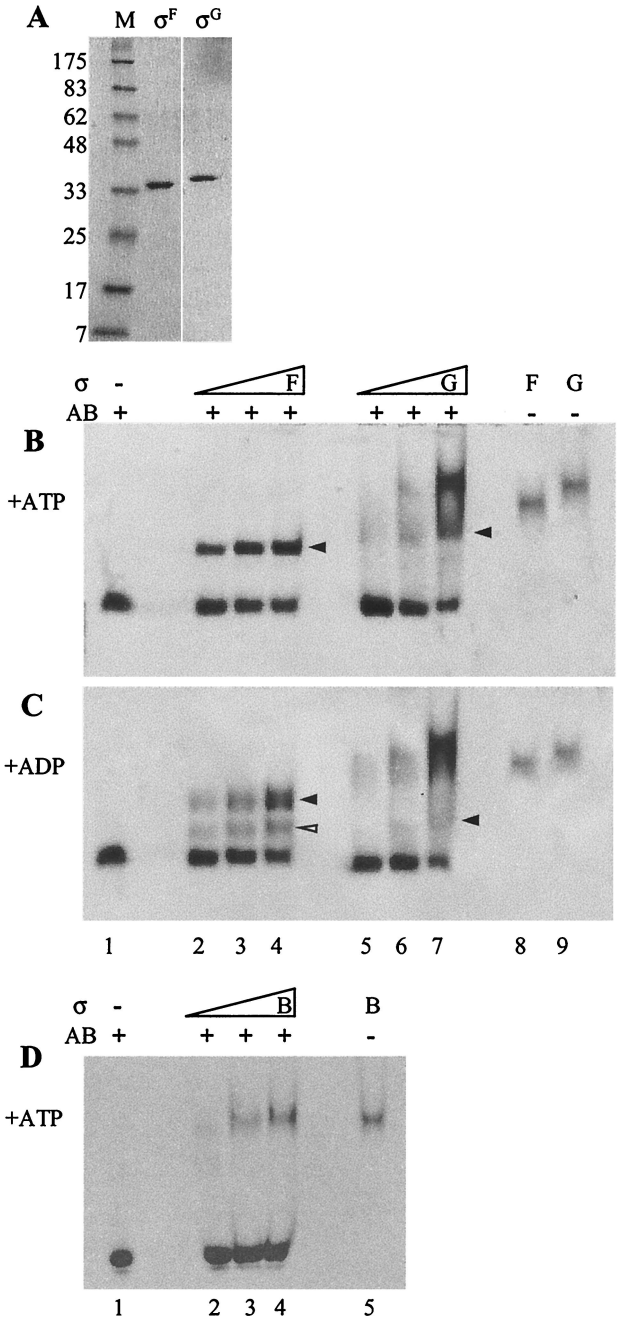

FIG. 1. SpoIIAB forms a complex with σ^G in the presence of nucleotide. (A) Purified σ^G and σ^F. Fractions containing the proteins were pooled after gel filtration and run on sodium dodecyl sulfate–12% polyacrylamide gels. M, molecular weight markers. (B and C) Increasing concentrations (1, 2, and 4 μM) of σ^F (lanes 2 to 4) or σ^G (lanes 5 to 7) were incubated with purified SpoIIAB (2 μM) in the presence of ATP (B) or ADP (C). (D) Increasing concentrations (1, 2, and 4 μM) of purified σ^B (lanes 2 to 4) were incubated with purified SpoIIAB (2 μM) in the presence of ATP. Running positions of the purified proteins alone are shown as follows: SpoIIAB (panels B to D, lanes 1), σ^F (panels B and C, lanes 8), σ^G (panels B and C, lanes 9), and σ^B (panel D, lane 5). Reactions were analyzed on 10% nondenaturing PAGE with the appropriate nucleotide added to the running buffer and were visualized by Coomassie blue staining. Complexes formed by σ and SpoIIAB are indicated by arrowheads.

with σ^G was in fact the weaker of the two. To confirm this finding, surface plasmon resonance was used to study the interaction between SpoIIAB and σ^G. Surface plasmon resonance has been used previously to look at the SpoIIAB-σ^F interaction (14). SpoIIAB (the ligand) was dialyzed overnight at 4°C into phosphate-buffered saline to which 1 mM DTT had been added and was immobilized onto a matrix in the flow cell of a sensor chip (CM5; Biacore), as described in reference 14. Purified σ^F and σ^G (the analytes) were dialyzed into binding buffer (50 mM Tris-HCl [pH 7.5], 50 mM KCl, 1 mM DTT, 3 mM $MgCl_2$) at 4°C overnight. Protein concentrations were determined by running samples on sodium dodecyl sulfate-PAGE gels stained with Sypro Orange (Amersham Bioscience) and comparing them with molecular weight markers of known concentrations. The dialyzed proteins were then diluted in binding buffer supplemented with 1 mM ATP to concentrations ranging from 0.1 to 5 μM. Using the Kinject function with a dissociation time of 150 ms, samples (20 μl) (containing 0.25 μM, 0.5 μM, 1 μM, and 2.5 μM protein) were injected into the flow cell with a flow rate of 20 μl min^{-1} in binding buffer containing ATP. After injection and dissociation, any undissociated analyte was removed by injection of 0.75 M NaCl in binding buffer without nucleotide until the sensorgram returned to the preanalyte baseline. Interactions with the ligand can be detected by an optical change at the gold surface onto which the matrix is attached. Dissociation from the ligand can also be measured through a reversal of the optical change once the flow of analyte has stopped. Analysis of the data was carried out using BIAevaluation software (Biacore); the sensorgram obtained from a control flow cell with no protein was subtracted from that obtained when the same concentration of σ factor was passed through the flow cell containing SpoIIAB. The resulting curves for at least two different concentrations of σ factor were then used to calculate the dissociation constant (K_d) for each σ factor. For the σ^F-SpoIIAB interaction, the K_d was found to be 8 nM, which is comparable to the value of 14 nM obtained previously (14). However, for the σ^G:SpoIIAB complex, a K_d value of 87 nM was obtained. Therefore, we can conclude that σ^G binds more weakly to SpoIIAB than does σ^F. The weaker interaction between the two proteins is probably responsible for the partial dissociation of the complexes during electrophoresis shown in Fig. 1. The fact that SpoIIAB forms a more stable complex with σ^F than with σ^G shows that the opposing regulation mechanisms of σ^G and σ^F activities are not modulated simply by different affinities of the complexes.

SpoIIAA dissociates the SpoIIAB:σ^G complexes. Another possible explanation for a mechanism by which σ^G could be held inactive by SpoIIAB at a time when σ^F is active is that SpoIIAA is unable to interact with and dissociate the SpoIIAB:σ^G complex. To test this possibility, we used fluorescence spectroscopy to observe complex formation between σ^G and SpoIIAB in real time (Fig. 2; results for three independent experiments are shown). This technique takes advantage of the fact that neither SpoIIAB nor SpoIIAA contains the highly fluorescent amino acid tryptophan but σ^G contains a single tryptophan residue (W198). Tryptophan fluorescence can be selectively excited at wavelengths of around 290 nm. Therefore, using fluorescence spectroscopy we were able to observe changes in the chemical environment of the single tryptophan residue in σ^G. Exciting σ^G at 290 nm gave a maximum fluorescence emission at 350 nm; hence, this wavelength was used to observe changes in fluorescence intensity. Control experiments confirmed that SpoIIAA and SpoIIAB exhibited very weak fluorescence and that this fluorescence was unchanged by the addition of ATP. The fluorescence of σ^G by itself changed only slightly when ATP was added. For Fig. 2, appropriate corrections were made to account for these fluorescence values.

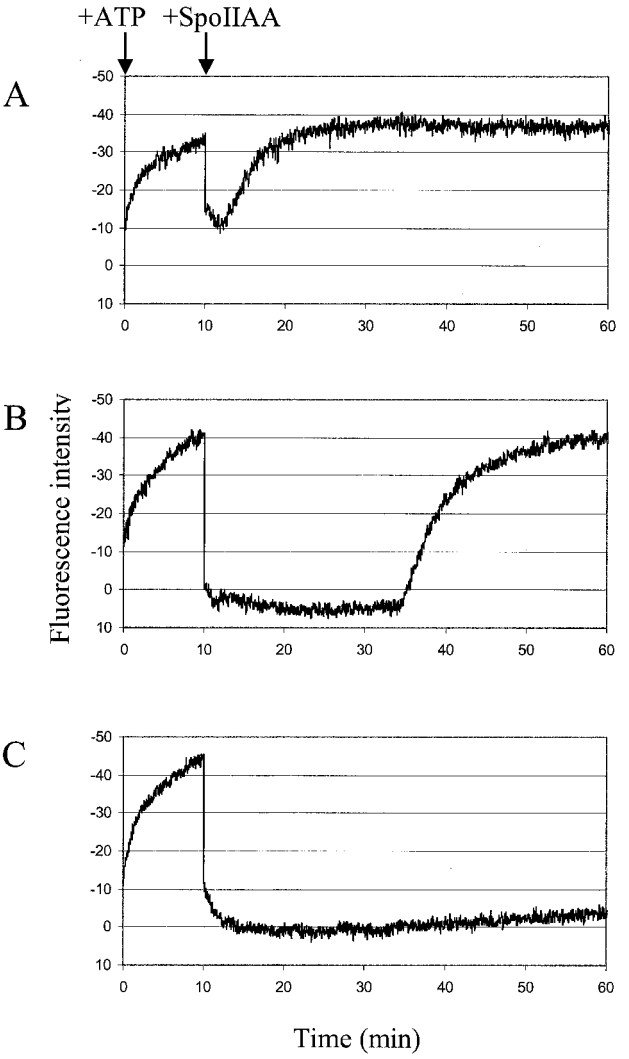

FIG. 2. SpoIIAA induces the release of σ^G from SpoIIAB:σ^G complexes. Fluorescence spectroscopy was used to measure changes in the fluorescence intensity of the single tryptophan in σ^G. The results of three independent experiments are shown in panels A to C. Purified σ^G (0.8 μM) and SpoIIAB (1.25 μM) were mixed, and at time zero, ATP (100 μM) was added to the solution and the fluorescence intensity of the sample was determined. Different concentrations of SpoIIAA (5 μM [A], 10 μM [B], and 15 μM [C]) were added to the samples 10 min later.

Solutions of σ^G (0.8 μM) and SpoIIAB (1.25 μM) were preequilibrated at 25°C for 5 min in 50 mM Tris-HCl (pH 7.5)–50 mM KCl–1 mM DTT–3 mM $MgCl_2$. The average basal fluorescence intensity was determined and set to 0. At time zero, ATP (100 μM) was added to the reaction mixture, which

immediately resulted in a substantial decrease in fluorescence intensity (Fig. 2A to C). Since this decrease in fluorescence intensity was much larger than that seen when ATP was added to σ^G alone, it can be attributed to changes in the chemical environment of the tryptophan in σ^G on binding to SpoIIAB. Thus, these results again show that σ^G and SpoIIAB interact with each other and that the binding occurs rapidly. Presumably, the tryptophan (W198) in σ^G is either directly involved in binding to SpoIIAB or it moves as a result of the interaction. The crystal structure of the SpoIIAB:σ^F complex was solved recently; however, the location of the corresponding tryptophan (W190) in σ^F is still unknown, as most of the σ^F protein was disordered and the fold could not be determined (4).

Using this system we were then able to determine whether SpoIIAA was able to disrupt the complex. At 10 min, the complex was challenged with 5 (Fig. 2A), 10 (Fig. 2B), and 15 (Fig. 2C) μM SpoIIAA. An excess of SpoIIAA was required to mimic the effect of the phosphatase SpoIIE, which replenishes the pool of SpoIIAA in vivo by dephosphorylation of SpoIIAA-P. Control experiments omitting each of the components in turn showed that the changes in fluorescence are due to effects on the tryptophan fluorescence of σ^G and therefore to changes in the level of the SpoIIAB(ATP):σ^G complex (data not shown). The addition of SpoIIAA caused a large, immediate increase in fluorescence intensity, which may be attributable to the release of σ^G from SpoIIAB. With 10 and 15 μM SpoIIAA, the values of the fluorescence intensity fell to the basal level observed for σ^G alone, indicating that SpoIIAA disrupts all of the SpoIIAB(ATP):σ^G complexes. Addition of 5 μM SpoIIAA led to a smaller increase in fluorescence intensity, suggesting that this concentration of SpoIIAA was not enough to disrupt all of the complexes.

After the initial increase in fluorescence, the reaction mixture containing 5 μM SpoIIAA gradually rose again to reach the level attained before the addition of the SpoIIAA. A similar rise was seen, albeit after a long delay, with 10 μM SpoIIAA (about 25 min) and 15 μM SpoIIAA (data not shown). We assume that these changes can be attributed to the phosphorylation of SpoIIAA by SpoIIAB (2, 5, 6, 16) and that the reaction proceeds in the presence of ATP until all of the SpoIIAA is used up; at this point the SpoIIAB is free to rebind to the sigma factor. (The experiments were repeated several times, giving similar results.) These results showed that SpoIIAA can efficiently disrupt SpoIIAB:σ^G complexes, releasing free σ^G, just as it disrupts SpoIIAB:σ^F complexes (2, 5, 6, 16).

Previous work has shown that SpoIIAB is capable of regulating σ^G activity under some circumstances (9, 10). However, three lines of evidence now suggest that SpoIIAB alone is not normally responsible for the temporal control of σ^G activity until after the completion of engulfment. First, we have shown that binding of SpoIIAB to σ^G is weaker than that to σ^F. Thus, in the presence of a mixture of σ^F and σ^G, binding to σ^F would be strongly favored, so σ^G would be released preferentially over σ^F. Second, we have shown that SpoIIAB:σ^G complexes are dissociated efficiently by the presence of nonphosphorylated SpoIIAA. Therefore, the same mechanism that helps to release σ^F activity soon after septation in the prespore, which involves formation of SpoIIAA by the action of SpoIIE phosphatase, would also promote the release of σ^G at that time. Third, the amount of free SpoIIAB (at the time σ^G begins to be synthesized) is probably small, as it has been shown recently that SpoIIAB is selectively degraded in the prespore (19). So far, we cannot exclude the possibility that another factor is required for the interaction of SpoIIAB and σ^G which then enables SpoIIAB to participate in the regulation of σ^G activation in vivo and which is missing from our in vitro studies.

Taken together, all of these results suggest that SpoIIAB may not be the primary effector responsible for temporal control over σ^G activation and that at least one other factor remains to be discovered. The challenge now is to identify this putative factor so that the mechanism responsible for developmental regulation of σ^G activity can finally be resolved.

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