The Kinase Activity of the Antisigma Factor SpoIIAB is Required for Activation as well as Inhibition of Transcription Factor \( \sigma^F \) During Sporulation in *Bacillus subtilis*

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The activity of the developmental transcription factor \( \sigma^F \) in the spore-forming bacterium *Bacillus subtilis* is controlled by SpoIIAB, which sequesters \( \sigma^F \) in an inactive complex. \( \sigma^F \) is released from the SpoIIAB-\( \sigma^F \) complex by the action of SpoIIAA, which triggers the dissociation of the complex. SpoIIAB is also a protein kinase that phosphorylates SpoIIAA on serine residue 58 (S58). This phosphorylation inactivates SpoIIAA and thus indirectly prevents the activation of \( \sigma^F \). Here, we report the identification of a patch of amino acid residues located in the vicinity of the adenosine nucleotide binding pocket of SpoIIAB that is required for the phosphorylation of SpoIIAA. A lysine substitution (E104K) at one of these residues (Glu104) markedly impaired the capacity of SpoIIAB to phosphorylate SpoIIAA *in vitro* as well as during sporulation. Kinetic analysis and evidence from the construction of alanine substitution mutants indicates that the side-chains of these amino acids could be contact sites for the SpoIIAA substrate during the phosphorylation reaction. Importantly, E104K and other kinase mutants blocked the activation of \( \sigma^F \) during sporulation. This is paradoxical, because a mutant of SpoIIAA (S58A) that cannot be phosphorylated is known to cause higher than normal levels of \( \sigma^F \) activity during sporulation. In resolution of this paradox, we present biochemical evidence indicating that SpoIIAA directly attacks the SpoIIAB-\( \sigma^F \) complex and that SpoIIAA is phosphorylated as a result of this reaction. Consistent with this idea, mutations impairing kinase function of SpoIIAB were found to be epistatic to a mutation causing the S58A substitution in SpoIIAA; that is, cells producing mutant forms of both proteins were blocked in the activation of \( \sigma^F \). We conclude that phosphorylation of SpoIIAA plays a dual role in the \( \sigma^F \) pathway, and that the kinase function of SpoIIAB is required for the activation as well as the inhibition of \( \sigma^F \) during sporulation.

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**Introduction**

The RNA polymerase sigma factor \( \sigma^F \) of the bacterium *Bacillus subtilis* is a developmental regulatory protein that directs gene transcription in a cell-specific fashion during the process of spore formation. The \( \sigma^F \) protein is produced shortly after entry into the sporulation process, but it does not become active until the developing cell (the sporangium) undergoes polar division (Stragier & Losick, 1996). The formation of a septum near one
pole of the sporangium creates a small progeny cell known as the forespore and a large cell known as the mother cell. The activity of $\sigma^F$ is confined to the forespore cell (Harry et al., 1995; Lewis et al., 1994; Margolis et al., 1991). Cell-specific activation of $\sigma^F$ is governed by a pathway involving the proteins SpoIIAB, SpoIIAA and SpoIIE (Alper et al., 1994; Diederich et al., 1994; Duncan et al., 1995, 1996; Duncan & Losick, 1993; Margolis et al., 1991; Min et al., 1993; Schmidt et al., 1990).

In the predivisional sporangium and in the mother cell, $\sigma^F$ is held in an inactive complex with SpoIAB, which is referred to as an antisigma factor (Duncan & Losick, 1993; Min et al., 1993). The $\sigma^F$ protein is liberated from the SpoIAB-$\sigma^F$ complex in the forespore due to the action of SpoIIAA, an anti-antisigma factor that binds to and antagonizes the action of SpoIAB (Alper et al., 1994; Diederich et al., 1994). The action of SpoIIAA is regulated by its interconversion between a phosphorylated and a dephosphorylated state (Arigoni et al., 1995; Duncan et al., 1995; Feucht et al., 1996; Lewis et al., 1996; Min et al., 1993). This interconversion is, in turn, governed by the opposing actions of a serine kinase and a phosphatase. The kinase is SpoIIAB, which is a dual function protein that is capable of binding to and inhibiting $\sigma^F$, and of phosphorlating and thereby inactivating SpoIIAA (Alper et al., 1994; Diederich et al., 1994; Magnin et al., 1996). The reverse reaction, the conversion of SpoIIAA-P to SpoIIAA, is catalyzed by the third member of the $\sigma^F$ regulatory pathway, SpoIIIE (Arigoni et al., 1996; Duncan et al., 1995; Feucht et al., 1996). Interestingly, the activity of the stress response transcription factor $\sigma^F$ of B. subtilis is governed in a similar but not identical fashion by homologs of SpoIIAB, SpoIIAA and SpoIIE (Benson & Haldenwang, 1993; Yang et al., 1996).

SpoIIAB has an inferred adenosine nucleotide binding pocket (the G-box; Stock et al., 1995) and can bind either ATP or ADP. In its ATP-containing form, SpoIIAB is able to bind to $\sigma^F$ (Alper et al., 1994) and to phosphorylate SpoIIAA (Min et al., 1993; Najafi et al., 1995). In the ADP-containing state, on the other hand, SpoIIAB is inactive as a kinase and is instead capable of forming a long-lived complex with SpoIIAA (Alper et al., 1994; Diederich et al., 1994; Magnin 1997). Thus, SpoIIAB and SpoIIAA are mutually antagonistic: ATP-containing SpoIIAB can inactivate SpoIIAA by covalent modification and SpoIIAA can inhibit ADP-containing SpoIIAB by sequestering it in an inactive SpoIIAA-SpoIIAB complex. The capacity of SpoIIAB to form alternative complexes with $\sigma^F$ and with SpoIIAA is known as partner switching (Alper et al., 1994; Diederich et al., 1994). Thus, the activity of $\sigma^F$ is determined by a switch that centers on SpoIIAB, which can inhibit $\sigma^F$ and inactivate SpoIIAA or can liberate $\sigma^F$ and form an alternative, inactive complex with SpoIIAA.

In the accompanying paper, we describe the results of a genetic screen for mutants of SpoIIAB that were blocked in the activation of $\sigma^F$ during sporulation (Garsin et al., 1998). Some of the amino acid substitutions identified in this screen, which were located in the N-terminal region of the protein, interfered with the capacity of SpoIIAB to bind to SpoIIAA but not to $\sigma^F$. Thus, the inability of these mutant proteins to interact with SpoIIAA explained the failure of $\sigma^F$ to escape from the SpoIIAB-$\sigma^F$ complex. Interestingly, however, amino acid substitutions were identified in two other regions of SpoIIAB that also prevented the activation of $\sigma^F$ but had no discernible effect on the capacity of the mutant protein to form a SpoIIAB-SpoIIAA complex in the presence of ADP. One of these regions is located in the central portion of SpoIIAB in close proximity to the inferred adenosine nucleotide binding pocket. Here, we present evidence indicating that these mutants define a patch of amino acid side-chains that are required for contacting SpoIIAA during the transient phosphorylation reaction but not for the formation of the stable SpoIIAB-SpoIIAA or SpoIIAB-$\sigma^F$ complexes. One such amino acid substitution is shown to impair the phosphorylation of SpoIIAA in vitro and to block both the activation of $\sigma^F$ and the phosphorylation of SpoIIAA during sporulation. This is paradoxical, because previous work has established that phosphorylation is indirectly responsible for inhibiting the activity of $\sigma^F$; as explained above, phosphorylation inactivates SpoIIAA and hence prevents the liberation of $\sigma^F$ from the SpoIIAB-$\sigma^F$ complex. We report and discuss the results of experiments that resolve the paradox and that led us to conclude that SpoIIAB-mediated phosphorylation is required for the activation of $\sigma^F$ as well as its inhibition.

Results
E104K is defective in phosphorylating SpoIIAA in vitro

In the accompanying paper we describe the isolation of SpoIIAB mutants that are blocked in the activation of $\sigma^F$ during sporulation (Garsin et al., 1998). Among the mutants so identified were amino acid substitutions in the N-terminal region of SpoIIAB that interfered with the formation of the SpoIIAB-SpoIIAA complex in the presence of ADP (Figure 1). However, mutants defective in the activation of $\sigma^F$ were additionally recovered in two other regions of SpoIIAB: a patch of amino acid residues located close to the inferred adenosine nucleotide binding pocket (the G-box) and the extreme C terminus of SpoIIAB. These mutants included two, E104K (which is near the G-box) and S139stop (which is near the C terminus), that caused a severe block in $\sigma^F$ activation and sporulation. Yet, neither E104K nor S139stop was measurably impaired in the formation of complexes with either SpoIIAA or $\sigma^F$.

What then is the nature of the defect caused by these mutant proteins? Because of the close proximity of E104 to the adenosine nucleotide binding
region (G-box), we wondered if an amino acid substitution at this position exerted its effect on the kinase activity of SpoIIAB. We addressed this question by comparing the capacity of wild-type and mutant SpoIIAB to phosphorylate SpoIIAA in the presence of ATP. The results indicated that E104K could phosphorylate SpoIIAA but did so significantly less effectively than did the wild-type protein (Figure 2). In other work (data not shown), S139stop was found to be indistinguishable from the wild-type in its capacity to carry out phosphorylation.

Next, we carried out a Michaelis-Menten kinetic analysis to determine whether ATP-bound E104K was defective in its capacity to bind to its substrate as reflected in the $K_m$ or in the phospho-transfer reaction as reflected in the $k_{cat}$. The results showed that E104K differed from the wild-type only modestly in $k_{cat}$ (by a factor of only two- to threefold), but exhibited a $K_m$ that was about 100-fold higher than that of wild-type SpoIIAB (Figure 3). These findings suggest that the kinase defect in E104K is principally due to a defect in its capacity to bind to SpoIIAA.

Because of the close proximity of the E104K substitution to the adenosine nucleotide binding pocket in SpoIIAB, it was conceivable that the defect in the kinase reaction was due to a reduced ability of SpoIIAB-E104K to bind ATP rather than to its protein substrate. Because ATP favors binding of SpoIIAB to $\sigma^F$ over ADP (Alper et al., 1994), we investigated the possibility that E104K interferes with the binding of ATP by comparing the affinity of wild-type and mutant SpoIIAB to $\sigma^F$ over a wide range of ratios of ATP to ADP. The results revealed no detectable difference between the two proteins (Paskowitz, 1997). The simplest interpretation of these results is that E104K does not interfere with the binding of ATP to SpoIIAB. Rather, the amino acid substitution is likely to interfere with the interaction of SpoIIAB with SpoIIAA during the kinase reaction.

A kinetic analysis of the kinase reaction has been reported by Magnin et al. (1997) and Najafi et al. (1997). These workers observed that the phosphorylation occurs in a biphasic fashion, an initial linear phase followed by a second, slower phase. Our measurements appear to correspond to the initial phase of the reaction and showed a simple linear dependence on time. The $k_{cat}$ we obtained at 21°C appears to be substantially higher than the values reported by these authors for their initial phase.

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**Figure 1.** Mutants of SpoIIAB. The rectangle represents the anatomy of SpoIIAB. The upper row of amino acid substitutions below the rectangle identifies mutants from the genetic screen of the accompanying paper that prevented the activation of $\sigma^F$ during sporulation but were unimpaired in their capacity to form complexes with SpoIIAA or $\sigma^F$ in vitro (Garsin et al., 1998). Substitutions causing a strong block in $\sigma^F$ activation and in sporulation are identified in bold type. The lower row indicates alanine substitutions that were created by site-directed mutagenesis. The left-hand bracket indicates the region of SpoIIAB as identified in the accompanying paper that contributes to the formation of the SpoIIAB-SpoIIAA and SpoIIAB-$\sigma^F$ complexes (Garsin et al., 1998). The right-hand bracket indicates the location of the region of SpoIIAB that is believed to be responsible for binding to SpoIIAA during the phosphorylation reaction.

**Figure 2.** E104K is defective in phosphorylating SpoIIAA. The phosphorylation reactions were carried out at 30°C for ten minutes in a 25 µl reaction volume containing 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM MgCl₂, 1 mM ATP, 1 mM DTT, 5 µl of radiolabelled SpoIIAA-containing cell lysate (Garsin et al., 1998), and purified SpoIIAB as indicated (concentration reflects dimer). The reactions were stopped by the addition of 25 µl of IEF loading solution and separated on an IEF gel (Arigoni et al., 1996) for two hours at 300 V. The gel was fixed in 10% TCA for ten minutes followed by two hours in 1% (v/v) TCA. The radiolabelled SpoIIAA was visualized by autoradiography.
E104K is defective in phosphorylating SpolIIA during sporulation

Next, we asked whether this defect in phosphorylation was manifest in vivo. Cells producing wild-type or mutant forms of SpolIIA were allowed to sporulate. Protein was then extracted from the cells and subjected to isoelectric focusing (IEF) to separate phosphorylated and unphosphorylated forms of SpolIIA. Finally, SpolIIA and SpolIIA-P were visualized by immunoblotting with anti-SpolIIA antibodies (Figure 4). Consistent with previous observations, conversion of SpolIIA to SpolIIA-P in cells producing wild-type SpolIAB commenced by about 90 minutes into sporulation (Arigoni et al., 1996; Feucht et al., 1996). In striking contrast, little or no phosphorylated SpolIIA was detected in cells producing SpolIAB-E104K either at 90 or 120 minutes. This finding is consistent with the results of the biochemical analysis and indicates that E104K is a kinase mutant.

For comparison, cells producing SpolIAB-T49K exhibited a subtle but significant defect in the conversion of SpolIIA to SpolIIA-P, as manifest by the timing as well as the extent of phosphorylation. As documented in the accompanying paper, the T49K amino acid substitution interferes with complex formation between ADP-containing SpolIAB and SpolIIA and is located in an N-terminal region of SpolIAB that is required for binding both to SpolIIA and to SpolIIA-P. Our present results suggest that the N-terminal region of SpolIAB additionally contributes to the interaction of ATP-containing SpolIAB with SpolIIA during the phosphorylation reaction. Conversely, however, the accompanying paper shows that SpolIAB-E104K was not defective in complex formation with SpolIIA in the presence of ADP. We conclude that the N-terminal region of SpolIAB is essential for SpolIIA phosphorylation in vivo.

Figure 3. Kinetic analysis of phosphorylation by SpolIAB and SpolIAB-E104K. Rates of $[γ-32P]$ incorporation into SpolIIA were measured for wild-type SpolIAB (●) and E104K (■) at varying concentrations of SpolIIA substrate and plotted as a Lineweaver-Burk plot. Data from three separate experiments with wild-type SpolIAB and SpolIAB-E104K were averaged and the brackets indicate the standard deviations. Because the $K_m$ for SpolIAB is so small, it was difficult to accurately measure by our methods and the value indicated should be considered an estimate.

Figure 4. SpolIAB-E104K and SpolIAB-T49K but not SpolIAB-S139stop are impaired in the phosphorylation of SpolIIA during sporulation. Phosphorylated and unphosphorylated forms of SpolIIA from sporulating strains of *B. subtilis* producing the indicated SpolIAB mutants were separated by isoelectric focusing and visualized by immunoblot analysis with anti-SpolIIA antibodies at the times indicated after the onset of sporulation.
region of SpoIIAB is needed both for complex formation and efficient phosphorylation, but that E104 is exclusively required for interaction with SpoIIAA during the phosphorylation reaction.

Lastly, we note that cells producing S139stop were not significantly different from the wild-type with respect to both the timing and the extent of phosphorylation of SpoIIAA.

Use of alanine substitution mutants to identify potential sites of contact with SpoIIAA during the phosphorylation reaction

Our genetic screen yielded three additional amino acid substitutions in the vicinity of E104K as well as a glycine substitution at E104 itself. All of these amino acid substitutions interfered with the activation of $\sigma^F$, although none did so as severely as E104K. Our provisional interpretation is that these amino acid substitutions identify a region of SpoIIAB that is responsible for binding to SpoIIAA during the phosphorylation reaction. Consistent with this idea, biochemical analysis showed that, like E104K, T98A was defective in phosphorylating SpoIIAA but was unimpaired in its capacity to form complexes with SpoIIAA and $\sigma^F$ (data not shown). If our interpretation is correct, then the side-chains of residues 96, 98, 99 and 104 could themselves be contact sites with SpoIIAA during phosphorylation or the L96Q, T99S and E104K substitutions could simply interfere with the capacity of ATP-containing SpoIIAB to interact with SpoIIAA. To distinguish between these possibilities, we created loss-of-side-chain (alanine substitution) mutants at residues 96, 99 and 104 (the T98A mutant was already a loss-of-side-chain substitution). The results showed that alanine substitutions at all three positions (four including position 98) resulted in a weak defect in the actuations at all three positions (four including substitution). The results showed that alanine substitution mutants at residues 96, 99 and 104 (the T98A mutant was already a loss-of-side-chain substitution) mutants at residues 96, 99 and 104 (the T98A mutant was already a loss-of-side-chain substitution). The results showed that alanine substitutions at all three positions (four including position 98) resulted in a weak defect in the actuations at all three positions (four including substitution). The results showed that alanine substitution mutants at residues 96, 99 and 104 (the T98A mutant was already a loss-of-side-chain substitution). The results showed that alanine substitutions at all three positions (four including position 98) resulted in a weak defect in the actuations at all three positions (four including substitution).

The E104K phosphorylation deficiency causes a block in $\sigma^F$ activation by blocking induced release

The attribution of a kinase defect to a SpoIIAB mutant (E104K) that is blocked in the activation of $\sigma^F$ creates a paradox. Previous work has established that phosphorylation is responsible for inactivating SpoIIAA (i.e., preventing it from interacting with SpoIIAB) and hence for indirectly inhibiting the activity of $\sigma^F$. Indeed, a mutant of SpoIIAA that is unable to be phosphorylated (created by an alanine substitution in SpoIIAA at the site of serine (S58) phosphorylation) causes higher than normal levels of $\sigma^F$ activity (Diederich et al., 1994). Why then does a SpoIIAB mutant that is defective in phosphorylating SpoIIAA exhibit greatly reduced levels of $\sigma^F$ activity?

A possible solution to the paradox comes from previous evidence indicating that the liberation of $\sigma^F$ from the SpoIIAB-$\sigma^F$ complex occurs by a direct and obligatory interaction of SpoIIAA with the SpoIIAB-$\sigma^F$ complex in a process known as induced release (Duncan et al., 1996). That is, the SpoIIAB-$\sigma^F$ complex is not greatly dynamic and does not undergo rapid cycles of dissociation and re-association. In other work the off rate was measured to be of the order of minutes (Magnin et al., 1997). Instead, release of $\sigma^F$ is induced by the interaction of unphosphorylated SpoIIAA with the SpoIIAB-$\sigma^F$ complex. Because SpoIIAB in the complex contains ATP, it was hypothesized that SpoIIAA would be phosphorylated as a result of interacting with SpoIIAB-$\sigma^F$ (Duncan et al., 1996). Knowing that ATP-containing SpoIIAB-E104K interacts poorly with SpoIIAA, we hypothesized that an E104K-$\sigma^F$ complex would be relatively immune to attack by SpoIIAA. If so, the failure of SpoIIAA to release $\sigma^F$ from such a mutant complex would explain the block of $\sigma^F$ activation observed in sporulating cells producing the mutant SpoIIAB protein.

To investigate this hypothesis, we formed complexes in the presence of ATP between wild-type and mutant forms of SpoIIAB, that had been radioactively labeled, and $\sigma^F$ that had been immobilized on a column of Affi-gel 10 (Biorad). We then attempted to disrupt the complexes by elution in the presence of buffer, purified $\sigma^F$ or purified SpoIIAA (Figure 5). In the case of wild-type SpoIIAB, neither buffer nor $\sigma^F$ caused a significant amount of radioactive protein to be eluted from the column. This confirms previous results and indicates that the SpoIIAB-$\sigma^F$ complex is relatively undynamic. In contrast, the application of SpoIIAA to the column caused the elution of a high proportion of the radioactive SpoIIAB. Moreover, when SpoIIAA in the eluate from the column was analyzed by isoelectric focusing (data not shown), it was observed that about half of the protein had been converted to SpoIIAA-P, a finding consistent with the idea that SpoIIAA becomes phosphorylated in the induced release reaction.

In comparison, in the case of complexes between radioactive E104K and immobilized $\sigma^F$, SpoIIAA was only marginally more effective than $\sigma^F$ in causing the elution of the radioactive mutant protein. As expected, little to none of the SpoIIAA in the eluate was in the phosphorylated form (data not shown). We conclude that the E104K-$\sigma^F$ complex undergoes the induced release reaction poorly and is relatively immune to attack by unphosphorylated SpoIIAA.

The experiment of Figure 5 also shows that SpoIIAA was inefficient in causing the release of radioactive T49K from column-bound complexes of the mutant protein with $\sigma^F$. Indeed, SpoIIAA
was less effective in eluting radioactive T49K protein than was σF. The modest level of radioactivity eluted from the column by σF could indicate that the T49K-σF complex is somewhat less stable and hence somewhat more dynamic than the corresponding wild-type complex. In any event, the results are fully consistent with the idea that efficient release of σF requires the direct interaction of SpoIIAA with the SpoIIAB-σF complex.

Finally, we used the induced release reaction to investigate further the nature of the defect caused by the SpoIIAB-S139stop mutant (Figure 5). The results indicated that complexes of σF with S139stop behaved similarly to complexes of σF with wild-type protein in their capacity to respond to SpoIIAA. We conclude that a defect in the induced release reaction is not the basis for the block in σF activation during sporulation observed in cells producing the S139stop protein. Thus, this mutant form of SpoIIAB, which causes a severe block in σF activation and sporulation, exhibited no measurable defect in its interaction with either SpoIIA or σF by any of the analyses employed in this and the accompanying report.

### Mutations causing the E104K and E104A substitutions in SpoIIAB are epistatic to a mutation causing the S58A substitution in SpoIIAA

In summary, we conclude that the dependence of σF activation on the phosphorylation of SpoIIAA occurs during the induced release reaction. If so, then the mutations causing the E104K and E104A substitutions should be epistatic to a mutation causing the S58A substitution in SpoIIAA. In otherwise wild-type cells, the presence of a mutant form of SpoIIA (S58A) that cannot be phosphorylated causes higher than normal levels of σF activity. We reasoned that in sporulating cells doubly mutant for both proteins, the presence of SpoIIAB-E104K or SpoIIAB-E104A in addition to SpoIIAA-S58A would block the activation of σF. That is, whether SpoIIAA is phosphorylated or not, σF would not be liberated from the E104K-σF or E104A-σF complex. Indeed, sporulating cells of a strain producing both SpoIIA-S58A and either SpoIIAB-E104K or SpoIIAB-E104A were as defective in the activation of σF as were strains producing wild-type SpoIIA and mutant SpoIIAB (data not shown).

### Discussion

SpoIIAB is capable of interacting with SpoIIA in two ways. In the presence of ADP, it forms a long-lived SpoIIAB-SpoIIAA complex. On the other hand, in the presence of ATP, it interacts transiently with SpoIIAA to catalyze the transfer of the γ-phosphate group of ATP to Ser58 of SpoIIAA, thereby generating ADP-containing SpoIIAB and SpoIIAA-P. Our present results and those of the accompanying paper (Garsin et al., 1998) show that both of these interactions are dependent on the N-terminal region of SpoIIAB. Thus, the substitution of Lys for Thr49 in SpoIIAB blocks the capacity of the mutant protein to form a complex with SpoIIA and significantly impaired its capacity to phosphorylate SpoIIA.

A principal contribution of the present investigation is the identification of a second region of SpoIIAB located in the central portion of the protein, near its inferred nucleotide-binding pocket (the G-box), that is important for the kinase reaction but not for complex formation. The defining residue of this region is E104, at which site a lysine substitution impairs phosphorylation of SpoIIA in vitro and prevents the conversion of SpoIIA to SpoIIA-P during sporulation. On the basis of kinetic analysis, we infer that the phosphorylation defect can be largely attributed to impaired binding of ATP-containing SpoIIAB-E104K to its SpoIIA substrate. Because an alanine substitution at E104

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**Figure 5.** Complexes of σF with SpoIIAB-E104K or SpoIIAB-T49K but not with SpoIIAB-S139stop are blocked in dissociation by SpoIIAA. The experiment was performed as described (Duncan et al., 1996) with the following modifications: the column matrix was covalently coupled to σF (final concentration 5 μM), and radiolabelled SpoIIAB from cell lysates containing either wild-type or mutant protein was allowed to bind in the presence of 1 mM ATP. Similar percentages of the different SpoIIAB mutants bound to the column. The columns were then either washed with buffer ( ), 5 μM σF ( ); or 5 μM SpoIIAA ( ). S139stop was tested in a separate experiment from E104 K and T49 K.
also impaired function (albeit not as severely), we infer that the E104 side-chain makes a positive energetic contribution to the binding of ATP-containing SpoIIAB to SpoIIAA during the phosphorylation reaction. Finally, because loss-of-side-chain substitutions at L96, T98 and T99 also impaired function, we infer that together with E104 these amino acid residues constitute a patch on the surface of SpoIIAB that is responsible for contacting SpoIIAA during the phosphorylation reaction.

The discovery that the block in $\sigma^F$ activation caused by SpoIIAB-E104K can be attributed to a defect in the phosphorylation of SpoIIAA is paradoxical. Previous work had established that phosphorylation inactivates SpoIIAA and thereby prevents the activation of $\sigma^F$. For example, cells producing SpoIIAA-S58A, which cannot be phosphorylated, exhibit greater than normal levels of $\sigma^F$ activity. Yet, our present results show that cells producing SpoIIAB-E104K are blocked in the activation of $\sigma^F$.

A solution to this paradox is provided by earlier work showing that the liberation of $\sigma^F$ from the SpoIIAB-$\sigma^F$ complex occurs by the direct attack of the complex by SpoIIAA and the speculation that SpoIIAA is phosphorylated in the induced release reaction (Duncan et al., 1996). In confirmation and extension of previous findings, the present investigation shows that the SpoIIAB-$\sigma^F$ complex is efficiently and rapidly dissociated by SpoIIAA, which undergoes phosphorylation in the process. The SpoIIAB-E104K-$\sigma^F$ complex, in contrast, is immune to attack and does not undergo rapid dissociation in the presence of SpoIIAA. These biochemical results together with the evidence showing that cells producing SpoIIAB-E104K are blocked in the activation of $\sigma^F$ and the phosphorylation of SpoIIAA during sporulation, lend strong support to the idea that the induced release reaction occurs in vivo and is an obligatory event in the activation of $\sigma^F$. It should be noted that as a result of the induced release reaction, SpoIIAB is released in an ADP-containing form (Figure 6C). This ADP-containing SpoIIAB could then interact with a second molecule of unphosphorylated SpoIIAA to create the long-lived SpoIIAB-SpoIIA complex, thereby sequestering the antisigma factor and preventing it from re-associating with $\sigma^F$.

Phosphorylation of SpoIIAA therefore plays a dual role in controlling the activity of $\sigma^F$. On one hand, phosphorylation results in the inactivation of SpoIIAA and hence the suppression of $\sigma^F$ activity (Figure 6B), while on the other hand, phosphorylation is involved in the liberation of $\sigma^F$ from the SpoIIAB-$\sigma^F$ complex and is hence needed for the activation of $\sigma^F$ (Figure 6C). This view of the dual role of phosphorylation predicts that the mutation causing the E104K substitution in SpoIIAB should be epistatic to a mutation causing the S58A substitution in SpoIIAA because induced release is the proximal event in the activation of $\sigma^F$. Indeed, we have shown that the effect of SpoIIAA-S58A, which by itself causes higher than normal levels of $\sigma^F$ activity, is reversed by the additional presence of SpoIIAB-E104K or SpoIIAB-E104A. That is, cells doubly mutant for both proteins fail to activate $\sigma^F$ during sporulation.

An alternative view of the role of phosphorylation in controlling the activity of $\sigma^F$ has been proposed (Magnin et al., 1997; Najafi et al., 1997). These workers have reported kinetic evidence that they interpret to indicate that in the course of every round of phosphorylation, the resultant ADP-containing SpoIIAB passes through a special state known as SpoIIAB*, which is slow to recycle to the ATP-containing form. This results in the activation of $\sigma^F$ because ADP-containing SpoIIAB* binds poorly to $\sigma^F$ (Najafi et al., 1997). Our induced release model and the “slow cycling” model (Magnin et al., 1997; Najafi et al., 1997) are not mutually exclusive, and indeed, our discovery that the kinase activity of SpoIIAB is required for the activation of $\sigma^F$ is not incompatible with slow cycling.

The amino acid sequence of SpoIIAB from B. subtilis has previously been compared to that from two other closely related species, B. megaterium and B. licheniformis (Park & Yudkin, 1997). The recent availability of a partial genome sequence for the distantly related endospore-forming bacterium Clostridium acetobutylicum provides an opportunity to test phylogenetically the significance of the residues identified in this and the accompanying paper as sites of interaction with SpoIIAA and $\sigma^F$ (Figure 7). Using a preliminary sequence of Clostridium spoilAB, we designed oligonucleotide primers and amplified the spoilAB gene of C. acetobutylicum by the polymerase chain reaction and determined its nucleotide sequence. Importantly, residues (R20 and N50) implicated as contact sites with SpoIIAA and $\sigma^F$ in the N-terminal region of SpoIIAB and the patch of amino acid residues (R20, N50, L96, T98, T99 and E104) implicated in binding to SpoIIAA during the phosphorylation reaction, are highly conserved in all four proteins. Indeed, five of the residues are conserved identically in all four proteins and the sixth, T99, is represented by a conservative substitution (serine) in the Clostridium protein. Moreover, R20, N50 and E104 fall into blocks of identically conserved amino acid sequences of 10, 14 and 9 residues in length, respectively. In lobo, the findings from phylogeny reinforce the view that the regions defined by our genetic and biochemical analysis play a fundamental role in the interactions of SpoIIAB with its two alternative partners.

Finally, we turn to the issue of the C-terminal region of SpoIIAB, where a missense mutant, S141G, and a nonsense mutant, S139stop, caused a partial and a strong block in $\sigma^F$ activation, respectively. Despite the severity of the phenotype of cells producing SpoIIAB-S139stop, the mutant protein exhibited no significant defect in its ability to dimerize or form complexes with SpoIIAA or $\sigma^F$, in its ability to phosphorylate SpoIIAA, or in its capacity to respond to SpoIIAA in the induced
release reaction. These observations suggest that there is a further, and apparently crucial, aspect to the function of SpoIIAB that has so far eluded detection. Whatever the nature of the mysterious function of the C-terminal tip of SpoIIAB, we note that the sequence at the extreme terminus is identical in nine out of 11 positions among the Bacillus proteins but that this conservation is not shared with SpoIIAB from the distant relative, C. acetobutylicum.
ammonium sulfate. Finally, pooled fractions containing SpoIIAB were loaded onto a G-75 or S-200 (Pharmacia) sizing column equilibrated in 20 mM Tris-HCl (pH 8.0), 175 mM NaCl, 1 mM EDTA, 1 mM DTT. The SpoIIAB-containing fractions were pooled, concentrated and stored in 50% (v/v) glycerol.

Quantitative kinetic measurements

Phosphorylation reactions were carried out at 21 °C in 500 μl reaction volumes containing 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 1 mM ATP plus 50 μM of [γ-32P]ATP (6000 Ci/mmol), 25 nM SpoIIAB. The SpoIIAA concentration (Garsin et al., 1998) was varied from 62.5 nM to 2 μM. Time-points were collected every 30 seconds from zero to four minutes after the addition of SpoIIAA. The reactions were terminated by adding 50 μl of the reaction to 850 μl of ice-cold 10% (v/v) trichloroacetic acid (TCA). The protein was precipitated overnight by the addition of bovine serum albumin to a final concentration of 1 mg/ml. The pellets were washed four times with 1 ml of 10% TCA and dissolved in 7 ml of scintillation fluor (Opti-Fluor, Packard) and counted in a liquid scintillation counter. The time-zero blank was subtracted from each value.

SpoIIAA immunoblot analysis

PY79 and congenic strains containing T49K, E104K and S139stop (Garsin et al., 1998) were sporulated by resuspension. After resuspension, 1 ml samples were pelleted and then resuspended in 100 μl of a lysis buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM MgCl₂, 0.3 mg/ml PMSF, 0.5 mg/ml lysozyme, 0.1 mg/ml DNaseI, and incubated at 37 °C for ten minutes. Isoelectric focusing sample buffer (100 mM Tris-HCl, pH 8.0, 1 mM DTT, 0.8 M ammonium sulfate, 1 mM EDTA, 0.05 M ammonium sulfate gradient of 0.8 M–0 M. SpoIIAB eluted at about 0.2 M ammonium sulfate. Finally, pooled fractions containing SpoIIAB were loaded onto a G-75 or S-200 (Pharmacia) sizing column equilibrated in 20 mM Tris-HCl (pH 8.0), 175 mM NaCl, 1 mM EDTA, 1 mM DTT. The SpoIIAB-containing fractions were pooled, concentrated and stored in 50% (v/v) glycerol.

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