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Interactions between a *Bacillus subtilis* Anti-σ Factor (RsbW) and Its Antagonist (RsbV)

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The activity of σ^B, a secondary σ factor of *Bacillus subtilis*, is primarily controlled by an anti-σ factor protein (RsbW) that binds to σ^B and blocks its ability to form an RNA polymerase holoenzyme (E-σ^B). Inhibition of σ^B by RsbW is counteracted by RsbV, a protein that is essential for the activation of σ^B-dependent transcription. When crude *B. subtilis* extracts were fractionated by gel filtration chromatography or electrophoresis through nondenaturing polyacrylamide gels, a complex composed of RsbW and RsbV that is distinct from the previously observed RsbW-σ^B complex was detected. In analogous experiments, RsbX, an additional regulator of σ^B-dependent transcription that is thought to act independently of RsbV-RsbW, was not found to associate with any of the other *sigB* operon products. Two forms of RsbV were visualized when crude cell extracts of *B. subtilis* were subjected to isoelectric focusing (IEF), with the more negatively charged RsbV species absent from extracts prepared from RsbW$^-$ strains. In vitro, RsbV became phosphorylated when incubated with ATP and RsbW but not with ATP alone. The phosphorylated RsbV species comigrated during IEF with the RsbW-dependent form of RsbV found in crude cell extracts. These results suggest that the modified RsbV, present in crude cell extracts, is phosphorylated. When gel filtration fractions containing RsbV-RsbW complexes or RsbV alone were subjected to IEF, only the unmodified form of RsbV was found associated with RsbW. The presumed phosphorylated variant of RsbV was present only in fractions that did not contain RsbW. The data support a model whereby RsbV binds directly to RsbW and blocks its ability to form the RsbW-σ^B complex. This activity of RsbV appears to be inhibited by RsbW-dependent phosphorylation.

σ^B is a secondary σ factor of *Bacillus subtilis*. RNA polymerase containing σ^B (E-σ^B) can be isolated from vegetatively growing and stationary-phase cells but not from cells that are undergoing sporulation (14–16). Null mutations in the σ^B structural gene (*sigB*) confer no obvious phenotype on strains which carry them (6, 13); however, genes which depend on σ^B for their expression are induced following heat shock or entry of *B. subtilis* into stationary phase (4, 7, 9, 17, 25). This pattern of σ^B-dependent gene expression suggests that σ^B plays a role, albeit a nonessential one, in the adaption of *B. subtilis* to certain environmental stresses.

sigB is the third gene of a four-gene operon that is transcribed from a σ^B-dependent promoter (13, 19). The operon's remaining three genes (*rsbV*, *rsbW*, and *rsbX*) have been shown in genetic and biochemical studies to encode regulators of σ^B (2, 3, 5, 8, 18). Null mutations in *rsbW* or *rsbX* result in dramatic increases in the expression of σ^B-dependent gene expression, while the loss of RsbV lowers this expression to levels comparable to that seen in mutant *B. subtilis* strains that lack σ^B itself (2, 8, 18). The requirement for RsbV in the expression of σ^B-dependent genes can be bypassed by mutations in *rsbW* but not *rsbX* (2, 8). Thus, the genetic evidence predicted RsbW to be the principal inhibitor of σ^B activity or synthesis, with RsbV being required only to modulate RsbW's inhibitory effects and RsbX functioning upstream of the RsbV-RsbW regulatory pair. Replacement of the *sigB* operon's σ^B-dependent promoter with the σ^A-dependent *SPAC* promoter maintains RsbV and RsbW's control of σ^B activity but eliminates the influence of RsbX (3, 8). These results imply that RsbV and RsbW are regulators of σ^B activity, while RsbX is likely to be a negative regulator of the *sigB* operon's σ^B-dependent promoter.

Biochemical experiments have suggested a mechanism for RsbW-dependent inhibition of σ^B (5). RsbW and σ^B were found to exist as a complex that could be immunoprecipitated from crude cell extracts (5). The association of σ^B with either RNA polymerase or RsbW in these extracts was mutually exclusive. Reconstitution experiments, in which purified σ^B and RsbW were added to core RNA polymerase in vitro, showed that incubation of RsbW with σ^B could block subsequent E-σ^B-dependent transcription (5). The data argued that RsbW functions as an anti-σ factor, binding to σ^B and preventing it from joining with RNA polymerase. Presumably, RsbV acts by facilitating the release of σ^B from the RsbW-σ^B complex.

σ^B is not the only *B. subtilis* σ factor that is regulated by an anti-σ factor. The sporulation-specific σ factor σ^F is the promoter-distal gene in an operon (*spoIIA*) whose upstream genes (*spoIIAA* and *spoIIAB*) encode products that are homologous to RsbV and RsbW (19). There is genetic evidence that the *spoIIAA* and *spoIIAB* products are potent regulators of σ^F activity, with the RsbW homolog (SpoIIAB) being an antagonist of σ^F and the RsbV homolog (SpoIIAA) counteracting SpoIIAB's negative effects (24). SpoIIAB, like RsbW, inhibits its target σ factor by a direct protein-protein interaction. In vitro studies have shown that purified SpoIIAB can physically associate with σ^F to the extent that the two proteins can be chemically cross-linked (12). In addition, incubation of σ^F with SpoIIAB inhibits σ^F's ability to initiate σ^F-dependent transcription in vitro (12, 22). In recent work, Alper et al. (1) have shown that SpoIIAA can associate with SpoIIAB, an interaction that may be necessary for σ^F activity. SpoIIAB has structural similarity to certain protein kinases (12, 22) and is able to cause phosphorylation of SpoIIAA in vitro (22). This

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TABLE 1. Bacterial strains used

B. subtilis strain	Relevant genotype	Reference
BSA105	$P_B\Delta 28::P_{SPAC}$, pTet-I	3
BSA106	$P_B\Delta 28::P_{SPAC}$, *rsbV312*, pTet-I	3
BSA107	$P_B\Delta 28::P_{SPAC}$, *rsbW313*, pTet-I	3
BSA116	$P_B\Delta 28::P_{SPAC}$, *sigB314*	5
BSA117	*rsbX::spec*	This study
BSA123	$P_B\Delta 28::P_{SPAC}$, *rsbX::spec*, pTet-I	3

and other results led Min et al. (22) to postulate that phosphorylation of SpoIIAA increases its affinity for SpoIIAB. Although SpoIIAA and SpoIIAB are principally regulators of σ^F, there is evidence that they also directly control the activity of σ^G, a second sporulation-specific σ factor whose transcription specificity is similar to that of σ^F (20, 23). Thus, the activities of at least two, if not three, *B. subtilis* σ factors are modulated by anti-σ factor proteins.

In this report, we describe interactions between the anti-σ^B factor RsbW and its antagonist, RsbV. Our results suggest that RsbV inhibits RsbW's ability to form an RsbW-σ^B complex by binding to RsbW. The binding of RsbV to RsbW appears to be influenced, at least in part, by the phosphorylation state of RsbV, with only the unphosphorylated form of RsbV associating with RsbW.

MATERIALS AND METHODS

Bacterial strains and culture media. Bacterial strains used are listed in Table 1. The null mutations created in the *rsbV*, *rsbW*, *sigB*, and *rsbX* genes (*rsbV312*, *rsbW313*, *sigB314*, and *rsbX::spec*) and the substitution of the *sigB* operon's promoter (P_B) by the *SPAC* promoter (P_{SPAC}) from pSI-1 (27) have been described (2, 3). Cells were grown in Luria broth (LB) (21), with the *SPAC* promoter induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

Gel filtration chromatography. Cells were harvested 1 h after induction of P_{SPAC} with IPTG, concentrated 200-fold in resuspension buffer (10 mM Tris HCl [pH 8.0], 10 mM EDTA, 50 mM NaCl, 1 mM $MgCl_2$, 0.3 g of phenylmethylsulfonyl fluoride per liter, 3 mM dithiothreitol), and mechanically disrupted as described previously (5). Then 2.5 ml of crude extract was loaded onto a 250-ml column of Sephacryl S-200 (Pharmacia) and eluted at 4°C, using resuspension buffer. After elution of the void volume, 2.5-ml fractions were collected. Aliquots (400 μl) of these fractions were precipitated with 2 volumes of ethanol and analyzed for RsbV, RsbW, σ^B, and RsbX by Western blotting (immunoblotting) (3). The column was calibrated by using an MW-GF-200 gel filtration molecular weight marker kit from Sigma, which includes proteins ranging from 12.4 to 200 kDa.

Immunological studies. The anti-σ^B, anti-RsbV, and anti-RsbW antibodies have been described elsewhere (3, 5). The anti-RsbX antibodies were prepared and analyzed for specificity as was done in the preparation of the anti-σ^B antibodies. Western blot analysis was performed by standard means (3).

IEF. Isoelectrofocusing (IEF) was performed in the horizontal Multiphor II electrophoresis system (LKB), using 5% acrylamide gels containing 8 M urea and a 1:1 mix of ampholytes with pH ranges of 2.5 to 5 and 4 to 6.5 (Pharmacia) at a final ampholyte concentration of 3%. The gel was prerun 10 min at 10 W, 5-μl samples were loaded, and electrophoresis was conducted at 25 and 35 W for 60 and 30 min, respectively, at a temperature of 15°C. The proteins were transferred by capillary action for 20 min at 37°C onto a nitrocellulose membrane and probed with antibodies as previously described (3).

In vitro phosphorylation. σ^B, RsbW, and RsbV, partially purified from *Escherichia coli*, were incubated in reaction mixtures (60 μl) containing 100 mM Tris HCl (pH 8.0), 0.5 mM EDTA, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$, 100 mM KCl, 0.5 mM dithiothreitol, 20 μg of phenylmethylsulfonyl fluoride per ml, 15% glycerol (10), and 10 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol) for 60 min at room temperature. The proteins were ethanol precipitated with bovine serum albumin (100 μg/ml) as the carrier and fractionated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Labeled proteins were visualized by autoradiography. The positions of RsbV and RsbW proteins on this gel were determined by a Western blot analysis of parallel lanes through which unlabeled RsbV and RsbW had been electrophoresed.

Nondenaturing PAGE. The separation gel for nondenaturing PAGE was 7.5% acrylamide in 375 mM Tris HCl (pH 8.9), with 250 mM Tris HCl (pH 6.7) used for the stacking gel. The reservoir buffer was 200 mM glycine–25 mM Tris HCl (pH 8.3) (26).

RESULTS

RsbV associates with RsbW. The *sigB* operon encodes σ^B and three additional proteins that modulate σ^B-dependent gene expression (19). At least two (RsbW and σ^B) of the *sigB* operon's four protein products (RsbV, RsbW, σ^B, and RsbX) associate to form a complex that can be resolved by gel filtration chromatography (5). Given the regulatory interactions that appear to take place among the *sigB* operon products (2, 3, 8), it seemed possible that additional associations exist among these proteins. The facility with which gel filtration allowed for the identification of the RsbW-σ^B complex and the current availability of antibody probes for all four *sigB* operon products prompted us to test whether we could use gel filtration to identify additional associations among these proteins. To generate sufficient *sigB* operon products for analysis, even in *B. subtilis* strains with mutations (e.g., *rsbV312* and *sigB314*) that normally block the operon's transcription, we chose to analyze strains in which the *sigB* operon's σ^B-dependent promoter had been replaced by the IPTG-inducible *SPAC* promoter (3).

A *B. subtilis* strain with a wild-type *sigB* operon, driven by P_{SPAC}, was first examined. The strain's P_{SPAC} promoter was induced with IPTG, and after 1 h, the culture was harvested for analysis. A crude cell extract was prepared and fractionated on Sephacryl S-200. The column fractions, containing proteins that were included within the gel matrix, were analyzed for their RsbV, RsbW, and σ^B content by Western blotting. Figure 1A illustrates the analysis of these fractions. The RsbW protein (18 kDa) (19) was present in most of the included fractions, overlapping the faster-eluting fractions containing σ^B (30 kDa) (6, 13) and the more slowly eluting fractions containing RsbV (12 kDa) (19). This order of elution parallels the relative molecular weights of the proteins; however, the specific fractions in which they eluted from the column were inconsistent with their monomer molecular mass. Based on calibration of the column with molecular mass standards, σ^B, RsbW, and RsbV were found in fractions where proteins of much larger size would be expected to elute.

When *B. subtilis* extracts are probed with our antibody reagents, σ^B is detected less readily than are the other operon products. We do not know whether this is a consequence of the

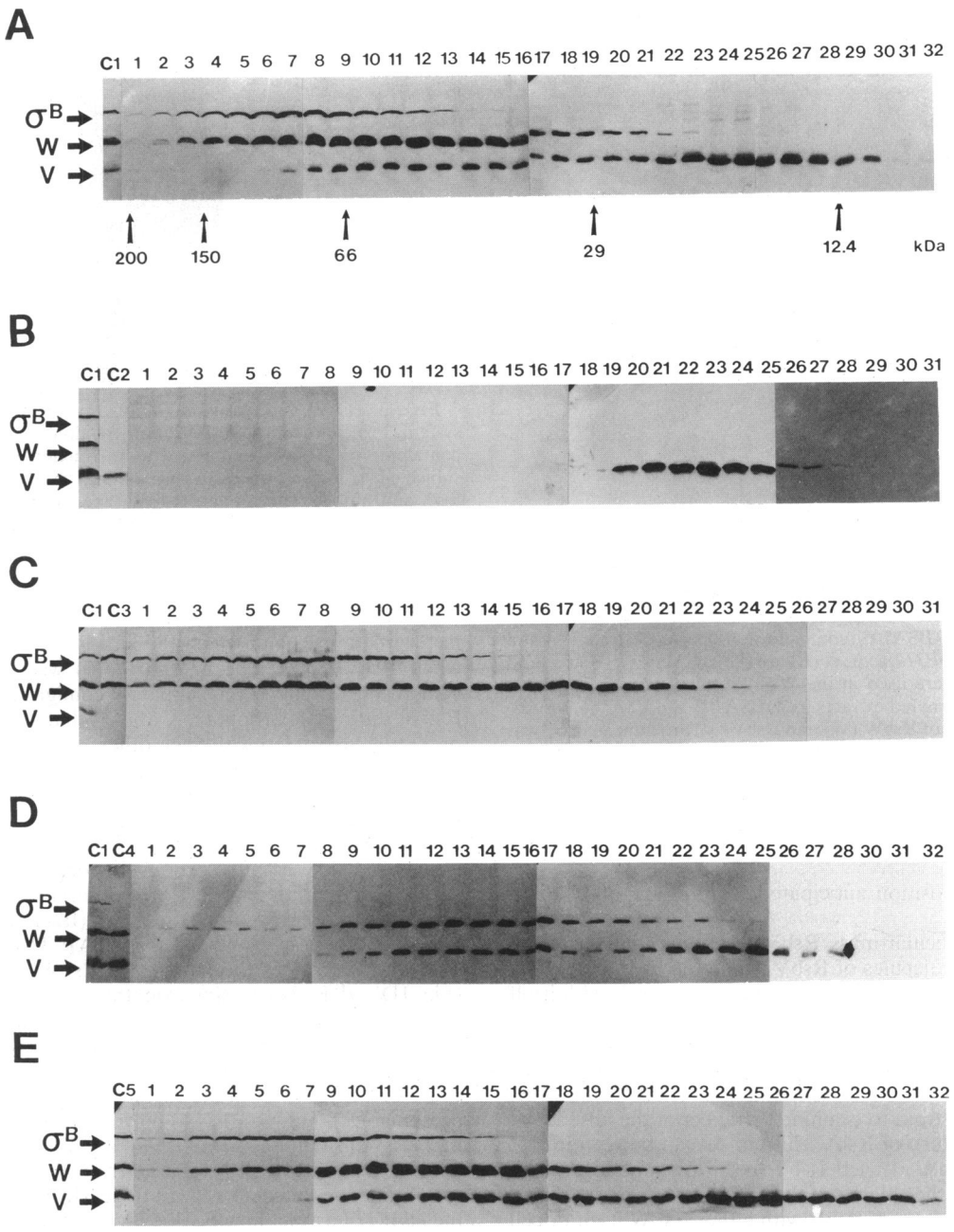

FIG. 1. Western blot analyses of gel filtration chromatography fractions of crude *B. subtilis* extracts. The *sigB* operon under the control of P_{SPAC} was induced by addition of IPTG to exponentially growing *B. subtilis* cultures (optical density at 540 nm of 0.2), and the cells were harvested 1 h later. Extracts were prepared and fractionated on a gel filtration column as described in Materials and Methods. Samples (400 μl) of the included fractions (2.5 ml) were ethanol precipitated and analyzed by Western blotting. Analyses of BSA105 (complete *sigB* operon) (A), BSA107 (*rsbW313*) (B), BSA106 (*rsbV312*) (C), BSA116 (*sigB314*) (D), and BSA123 (*rsbX::spec*) (E) fractions are shown. Anti-σ^B, -RsbW, and -RsbV monoclonal antibodies were used in the Western blot analyses. Lanes C1, C2, C3, C4, and C5 are unfractionated extracts from BSA105, BSA107, BSA106, BSA116, and BSA123, respectively. The positions of σ^B, RsbW (W), and RsbV (V) are indicated. The analyses start after the elution of the proteins excluded from the column and fractions to which the same number has been assigned can be compared. The arrows under panel A indicate the elution peaks of the indicated molecular mass markers.

relative abundance of these proteins in the extracts or the efficiency with which our reagents can detect them. For the Western blot analyses depicted in Fig. 1, sufficient extract was used to allow visualization of σ^B. This amount of extract contained so much RsbV and RsbW, however, that our assay became saturated for some fractions. We therefore repeated our analysis, using ≤10% of the material shown in Fig. 1. Figure 2A, which illustrates this analysis on some of the fractions depicted in Fig. 1A, shows that RsbV eluted in two peaks. The faster-eluting peak (fractions 10 to 12) occurred at a position where proteins of 50 to 60 kDa would be expected to exit the column, while the retarded peak (fractions 23 to 25)

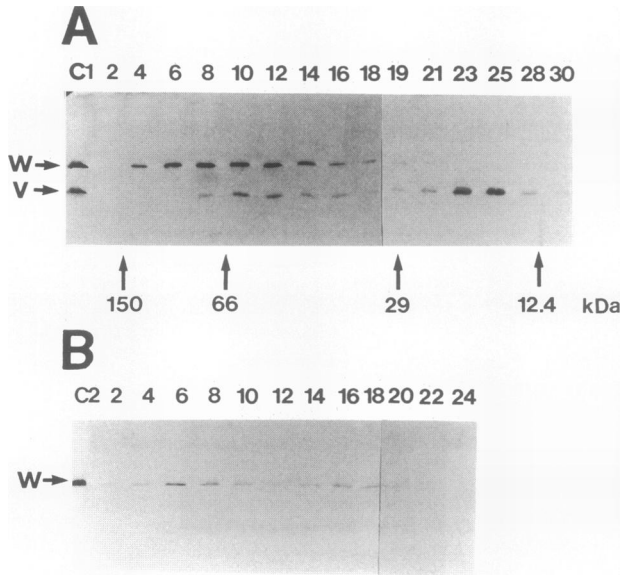

FIG. 2. Western blot analyses of gel filtration fractions using reduced protein amounts. Samples of the fractionated extracts described in Fig. 1 were precipitated and analyzed as in that figure. Analyses of 40 μl of BSA105 (complete *sigB* operon) extract (A) and 15 μl of BSA106 (*rsbV312*) extract (B) are shown. Anti-σ^B, -RsbW, and -RsbV antibodies were used in the Western blot analyses. Lanes C1 and C2 are unfractionated extracts from BSA105 and BSA106, respectively. The positions of RsbW (W) and RsbV (V) are indicated. σ^B was not detected in this analysis. The fractions are numbered as in Fig. 1. The arrows under panel A indicate the elution peaks of the indicated molecular mass markers.

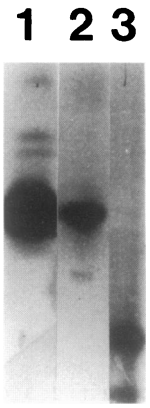

FIG. 3. RsbW and RsbV migrations in a nondenaturing PAGE system. Proteins from a gel filtration fraction of *B. subtilis* BSA117 containing σ^B, RsbW, and RsbV as in fraction 9 of Fig. 1A were subjected to native nondenaturing PAGE, transferred to nitrocellulose, and probed with an anti-RsbW (lane 1) or anti-RsbV (lane 2) monoclonal antibody. RsbV, expressed and purified from *E. coli* (4), was included and probed with an anti-RsbV antibody (lane 3).

had the elution position anticipated for proteins of 16 to 20 kDa.

The lower-molecular-mass RsbV fraction probably represents monomer molecules of RsbV, while the high-molecular-mass forms likely correspond to multimers of RsbV with itself or other proteins. The coincidence of RsbV with RsbW in these fractions (Fig. 2A) leaves open the possibility that RsbW is part of an RsbV-RsbW mixed multimer. To test this idea, we repeated the experiment, fractionating a crude cell extract from an $RsbW^-$ strain to examine the effect of the absence of RsbW on the pattern of RsbV elution. As can be seen in Fig. 1B, the loss of RsbW restricts RsbV to the low-molecular-mass fractions. Thus, the higher-molecular-mass form of RsbV represents associations of RsbV with either RsbW itself or cell components dependent on RsbW. A mixed RsbV-RsbW dimer would have a predicted molecular mass of 30 kDa. Thus, if the higher-molecular-mass form is a multimer of RsbV and RsbW, its elution position (50 to 60 kDa) suggests a tetramer composed of two molecules of RsbV and two of RsbW.

The idea that RsbV and RsbW are physically associated in the fractions where they coelute from the gel filtration column was examined by electrophoresis of fractions that contained RsbV and RsbW, or RsbV alone, on nondenaturing polyacrylamide gels and probing the resulting separated proteins with anti-RsbV or anti-RsbW antibodies. Figure 3 illustrates a Western blot of the proteins contained in a gel filtration fraction equivalent to fraction 9 of Fig. 1A. The anti-RsbW antibody reacted with a prominent protein band in the extract (Fig. 3, lane 1). The anti-RsbV antibody reacted with a protein band of the same mobility as that detected by the anti-RsbW antibody (Fig. 3, lane 2). This band was distinct from the faster-migrating band which the anti-RsbV antibody revealed when RsbV purified from *E. coli* (5) was analyzed in this system (Fig. 3, lane 3). The apparent comigration of RsbV with RsbW during electrophoresis argues that these proteins are physically associated with each other.

The RsbW protein, present in the wild-type extract, elutes as a broad peak that overlaps both the σ^B- and RsbV-containing fractions (Fig. 1A and 2A). We had previously documented complexes between RsbW and σ^B (5). Although the profile of potential RsbW-σ^B associations is complicated by putative RsbW-RsbV multimers, RsbW-σ^B multimers are likely to be present in the early-eluting fractions (fractions 2 to 13) that contain both RsbW and σ^B (Fig. 1A). The absence of σ^B significantly reduces the amount of RsbW in these fractions (Fig. 1D). σ^B is also missing from these fractions of the extract prepared from the *rsbW313* strain (Fig. 1B). In this case, the absence of σ^B is complicated by secondary effects of the *rsbW313* allele on the accumulation of σ^B (3). Although we could detect some σ^B in the excluded fractions of this column, presumably bound to RNA polymerase (data not shown), there is substantially less σ^B (~10%) present in *rsbW313* extracts than in extracts prepared from $RsbW^+$ strains. Thus, the absence of detectable σ^B in the early included fractions of the *rsbW313* extract may be due to both the lower levels of σ^B and the lack of RsbW-σ^B complexes.

In the absence of RsbV (Fig. 1C and 2B), substantial amounts of RsbW remain in the σ^B-containing fractions and a portion of the RsbW is shifted to a more slowly eluting position (compare Fig. 1A with Fig. 1C and 2A with Fig. 2B). Given that RsbW-σ^B can be coimmunoprecipitated as a complex from similar extracts (5) and that the abundance of RsbW in the early-eluting fractions is dependent on the presence of σ^B (Fig. 1A versus D), it is reasonable to speculate that early-eluting RsbW is associated with σ^B. The peak of RsbW (fraction 6; Fig. 2B) in these fractions corresponds to the elution position of a protein of 90 to 100 kDa. An RsbW-σ^B heterodimer would be expected to have a mass of 48 kDa. Thus, unless other unknown proteins are part of the complex, the apparent size of the putative RsbW-σ^B complex is consistent with an $RsbW_2$-$\sigma^B{}_2$ tetramer (96 kDa).

It is unlikely that the fractions (fractions 7 to 13) that contain

RsbV, RsbW, and σ^B represent aggregations of all three proteins in a single complex. Elimination of RsbV does not influence the amount or distribution of the putative σ^B-RsbW complex (Fig. 1C), and the absence of σ^B does not alter the pattern of the putative RsbV-RsbW complex (Fig. 1D). The simplest interpretation of these results is that RsbW associates independently with either RsbV or σ^B and the presence of the three proteins in overlapping fractions is due to the similar sizes of their individual complexes.

As mentioned above, the RsbW profile shifts in the absence of RsbV to the lower-molecular-weight region of the included proteins (Fig. 1C and 2B). Instead of a single broad peak centered around fraction 10 (Fig. 2A), which probably includes RsbW-σ^B and RsbW-RsbV complexes, RsbW elutes as two peaks in the *rsbV312* extract (Fig. 2B). Aside from the putative RsbW-σ^B peak (Fig. 2B, fraction 6), there is a peak at a position (fraction 16) corresponding to the elution position of a 36-kDa protein. Monomeric RsbW (18 kDa) would be expected to exit the column around fraction 24. It is possible that the RsbW eluting from the column at the 36-kDa position represents RsbW homodimers. In support of this notion, dimers of the RsbW homolog SpoIIAB have been reported (12).

RsbX does not affect association of RsbW with σ^B or RsbV. RsbX is the third regulator of σ^B-dependent transcription (2, 8, 18). Genetic experiments indicate that RsbX functions upstream of the RsbV-RsbW regulatory pair and is likely to be involved in the regulation of the *sigB* operon's σ^B-dependent promoter (3). As a further test of the independence of the RsbX and RsbV-RsbW systems, we examined whether the presence or absence of RsbX would affect the associations of RsbV, RsbW, and σ^B. To do this, we prepared an extract from an RsbX$^-$ *B. subtilis* strain and compared the gel filtration patterns of the RsbV, RsbW, and σ^B proteins in this extract (Fig. 1E) with that of an extract that contained RsbX (Fig. 1A). As can be seen in the figures, the gel elution patterns of RsbV, RsbW, and σ^B in the two extracts were indistinguishable.

We next probed gel filtration fractions with anti-RsbX antibody to determine whether RsbX is present in *B. subtilis* extracts as a free protein or, like the other *sigB* operon products, associated with other components of the extract. Figure 4A represents the extract examined in Fig. 1A probed with anti-RsbX antibody. The bulk of the RsbX (22 kDa) (19) eluted in fractions where proteins of 23 to 25 kDa would be expected to exit the column. This material likely represents monomeric molecules of RsbX. There was a small amount of RsbX in the column's excluded volume (not shown) which trailed into the leading included fractions (fraction 1 to 9). The presence of RsbX in the excluded volume probably reflects its association with large-molecular-weight components. The specificity or relevance of these associations is not known.

Inasmuch as the RsbX exited the column in fractions that also contained RsbV, we repeated the RsbX analysis in a *B. subtilis* strain that lacked RsbV to verify that the presence of RsbX in these fractions is independent of RsbV. Figure 4B illustrates a gel filtration fractionation of an extract from an RsbV$^-$ strain that was probed with anti-σ^B, anti-RsbW, and anti-RsbX antibodies. As expected, the RsbX pattern was unaffected by the loss of RsbV. The independence of the RsbX elution profile from that of σ^B, RsbV, and RsbW and the absence of an RsbX effect on the RsbW-RsbV-σ^B gel filtration pattern support the notion that RsbX is an independent regulatory element that is unlikely to function by directly interacting with σ^B or the RsbV-RsbW regulatory pair.

Two RsbV forms are distinguishable by IEF. Since σ^B-dependent transcription is inducible in response to environmental or physiological cues, one or more of the proteins that regulate σ^B activity is likely to exist in two interconvertible forms. If such a putative modification altered the protein's isoelectric qualities (pI), it could result in a change of the protein's position on an IEF gel. To explore this possibility, crude cell extracts from strains containing intact *sigB* operons, as well as extracts from strains lacking specific *sigB* operon products, were subjected to IEF using a horizontal gel system that allows comigration of several different samples at once and were probed for *sigB* operon products by Western blot analyses. Using this system, only a single species of RsbW was reproducibly observed (data not shown); however, two prominent bands and, occasionally, a fainter third band of RsbV protein were detected in extracts prepared from strains with a wild-type *sigB* operon or strains that failed to synthesize σ^B or RsbX (Fig. 5, lanes 1, 4, and 5, respectively). The identity of these proteins as the product of the *rsbV* gene was verified by their absence from an extract of an RsbV$^-$ strain (Fig. 5, lane 3). Interestingly, only a single RsbV band was found in an extract prepared from an RsbW$^-$ strain (Fig. 5, lane 2). Apparently, the presence of the additional RsbV bands depends on RsbW. From the relative intensity with which the anti-RsbV antibody reacted with the two prominent forms of RsbV, they are present in comparable amounts, with the more electronegative species being approximately twice as abundant as the other. We conclude that there are at least two forms of RsbV in *B. subtilis*, and the second form is likely to be created in a process that depends on RsbW.

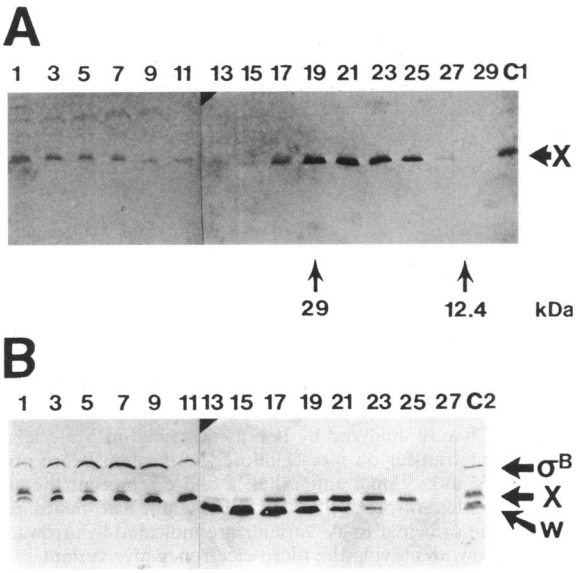

FIG. 4. Western blot analysis of RsbX in gel filtration fractions. Samples of fractions from the gel filtration chromatography of BSA105 (wild type), analyzed in Fig. 1A (A) or BSA106 (*rsbV312*), analyzed in Fig. 1C (B), were subjected to Western blot analysis using anti-RsbX as the probe in panel A and anti-σ^B, -RsbW, and -RsbX as the probes in panel B. Lanes C1 and C2 are unfractionated extracts of BSA105 and BSA106, respectively. The bands corresponding to σ^B, RsbW (W), and RsbX (X) are indicated.

RsbW phosphorylates RsbV in vitro. RsbV and RsbW are homologous to two other *B. subtilis* gene products, SpoIIAA and SpoIIAB, respectively (19). SpoIIAA and SpoIIAB are regulators of σ^F, a sporulation-specific σ factor, which they control in a fashion analogous to the regulation of σ^B by RsbV and RsbW (12, 24). The RsbW homolog SpoIIAB has been

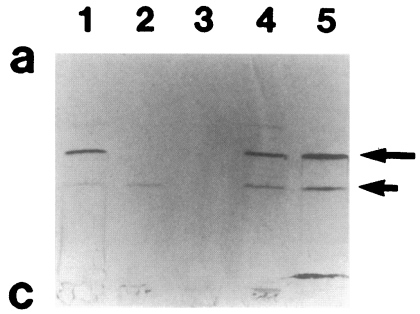

FIG. 5. IEF of crude *B. subtilis* extracts. Aliquots of crude extracts from BSA105 (complete *sigB* operon) (lane 1), BSA107 (*rsbW313*) (lane 2), BSA106 (*rsbV312*) (lane 3), BSA116 (*sigB314*) (lane 4), and BSA123 (*rsbX*::*spec*) (lane 5) prepared for the gel filtration fractionations of Fig. 1 were analyzed by IEF as described in Materials and Methods. After transfer on nitrocellulose, the proteins were probed with anti-RsbV monoclonal antibodies. a and c represent the anode and cathode poles, respectively, of the IEF unit. The bands corresponding to the principal RsbV variants are indicated by arrows, with the longer arrow identifying the more electronegative variant.

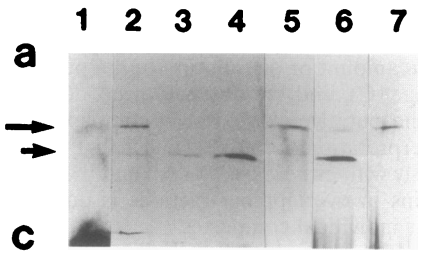

FIG. 7. IEF analysis of RsbV from gel filtration fractions and in vitro phosphorylation reactions. Lane 1 contains RsbV labeled in an in vitro reaction with [γ-^{32}P]ATP and run on the IEF gel, with the position of the labeled protein identified by autoradiography. Lanes 2 and 3 contain crude extracts of BSA105 (both forms of RsbV) and of BSA107 (*rsbW313*; only a single form of RsbV), respectively. Lanes 4 to 7 contain aliquots (200 μl) of fractions containing either RsbV-RsbW complexes or RsbV monomers. The samples in lanes 2 to 7 were ethanol precipitated, subjected to IEF, transferred to nitrocellulose, and probed with an anti-RsbV monoclonal antibody. Lane 4, fraction 11 (Fig. 1A) (RsbV-RsbW complex); lane 5, fraction 25 (Fig. 1A) (RsbV monomers); lane 6, fraction 10 (Fig. 1D) (RsbV-RsbW complex); lane 7, fraction 25 (Fig. 1D) (RsbV monomers). The bands corresponding to the principal RsbV variants are identified with arrows as in Fig. 5.

reported to have amino acid sequence elements in common with conserved elements of protein kinases and can phosphorylate the other member of its regulatory pair (SpoIIAA) in vitro (12, 22). These observations, in conjunction with the presence of RsbW-dependent alternative forms of RsbV in crude *B. subtilis* extracts, prompted us to examine whether RsbV and RsbW could be phosphorylated in vitro. RsbV, RsbW, and σ^B, expressed in and purified from *E. coli*, were incubated singly or in combination in a reaction mixture (10) containing [γ-^{32}P]ATP. The protein components of the reactions were then separated by SDS-PAGE. As can be seen in Fig. 6, lanes 1 to 3, neither RsbV nor RsbW nor σ^B became labeled when each was independently incubated with [γ-^{32}P]ATP. However, RsbV was labeled when coincubated with RsbW (Fig. 6, lane 4). Neither a mixture of σ^B and RsbV (Fig. 6, lane 5) nor a mixture of σ^B and RsbW (Fig. 6, lane 6) resulted in the labeling of any proteins. The labeling of RsbV by [γ-^{32}P]ATP probably involves the transfer of a phosphate group from ATP to RsbV. No labeling of RsbV was observed when [α-^{32}P]ATP was the labeled nucleotide (11). RsbW

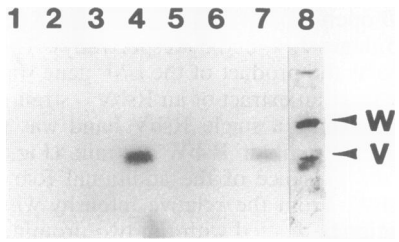

FIG. 6. In vitro phosphorylation of RsbV. Partially purified RsbV, RsbW, and σ^B were incubated with [γ-^{32}P]ATP separately or in mixtures and subjected to SDS-PAGE, and the labeled proteins were detected by autoradiography as described in Materials and Methods. The incubated proteins are RsbV (lane 1), RsbW (lane 2), and σ^B (lane 3) and mixtures of RsbV and RsbW (lane 4), RsbV and σ^B (lane 5), σ^B and RsbW (lane 6), and RsbV, RsbW, and σ^B (lane 7). The amount of RsbV, RsbW, and σ^B present was held constant in each reaction. To visualize the positions of RsbV (V) and RsbW (W), a mixture of these proteins was loaded on the same gel, transferred to nitrocellulose, and probed with both anti-RsbV and anti-RsbW antibodies (lane 8).

therefore either facilitates the autophosphorylation of RsbV or, as is more likely, functions as a protein kinase. The RsbW-dependent labeling of RsbV still occurred when σ^B was added to the mixture (Fig. 6, lane 7) but to a reproducibly lower extent. This reduction in RsbV labeling may be due to the formation of RsbW-σ^B complexes which could decrease the level of free RsbW; however, we have not excluded the possibility that an unknown contaminant in our σ^B preparation is responsible for this phenomenon.

The phosphorylation of RsbV in vitro by an RsbW-dependent mechanism suggests that the alternative form of RsbV that is observed following IEF of RsbW$^+$ extracts may be created by a similar reaction. We therefore compared the IEF properties of the phosphorylated form of RsbV with those of the RsbV proteins found in crude *B. subtilis* extracts. As can be seen in Fig. 7, the labeled RsbV protein (Fig. 7, lane 1) comigrated with the more electronegative form of RsbV (Fig. 7, lane 2) that is absent from the RsbW extract (Fig. 7, lane 3). Given that RsbV phosphorylated in vitro by an RsbW-dependent reaction has the same IEF characteristics as the RsbW-dependent form of RsbV that occurs in vivo, we speculate that this second in vivo form of RsbV represents a subpopulation of RsbV molecules that were phosphorylated, presumably by RsbW.

Phosphorylated RsbV is not associated with RsbV-RsbW complexes. Fractionation of *B. subtilis* extracts by gel filtration resolved two apparent peaks of RsbV protein: one associated with RsbW and a second without any obvious association (Fig. 1 and 2). IEF experiments also revealed two forms of RsbV which were distinguishable by their pIs (Fig. 5 and 7). We wished to determine whether these two observations could be related and examined the IEF properties of the RsbV present in various gel filtration fractions. The results of this examination are illustrated in Fig. 7 and demonstrate a clear segregation of the two forms of RsbV: the RsbV that was associated with RsbW (Fig. 1A, fraction 11; Fig. 1D, fraction 10) migrated to the position of unmodified RsbV (Fig. 7, lanes 4 and 6, respectively), while the RsbV that eluted in fractions without RsbW (Fig. 1A, fraction 25; Fig. 1D, fraction 25) consisted almost entirely of the putative phosphorylated version (Fig. 7,

lanes 5 and 7, respectively). These results argue that the state of RsbV, detected by IEF, affects its ability to associate with RsbW. That is, it appears that only the unmodified version of RsbV is able to bind to RsbW.

DISCUSSION

The principal regulator of σ^B activity is a σ factor-binding protein (RsbW) that sequesters σ^B into a complex wherein it is unavailable to RNA polymerase (5). At least a portion of σ^B is released from RsbW when σ^B-dependent transcription is induced at the onset of stationary phase by a process that depends on RsbV, a putative antagonist of RsbW (2, 3, 8). In this report, we present evidence that RsbV opposes RsbW's block on σ^B by directly associating with RsbW. Extracts in which the *sigB* operon is induced to synthesize its products to a high level display at least two RsbW-containing complexes that can be resolved by gel filtration chromatography. One contains the previously observed RsbW-σ^B complex, while the second contains RsbW and RsbV. The possibility that the coelution of RsbW and RsbV from the gel filtration column is due to an association between these two proteins is suggested by the observation that the gel filtration elution profiles of both of these proteins are altered when extracts lacking the other protein are analyzed (e.g., Fig. 1A versus B and Fig. 2A versus B) and that RsbV and RsbW from coeluting fractions comigrate when subjected to electrophoresis on nondenaturing gels.

From their elution positions from the gel filtration column, the RsbW-σ^B and RsbW-RsbV complexes are potential tetramers composed of two molecules of RsbW and two molecules of either σ^B or RsbV. In the homologous *spoIIA* system, apparent tetramers of SpoIIAB-σ^F were detected during SDS-PAGE of cross-linked proteins (12). Although the apparent sizes of the RsbW-σ^B and RsbW-RsbV complexes are consistent with these proteins being tetramers, we cannot exclude the possibility that their early elution from the column is due to additional associations with unknown proteins. The existence of RsbW dimers was suggested by the gel filtration profile of the $RsbV^-$ extract (Fig. 2B), in which a second peak of RsbW was evident in those fractions (fractions 14 to 18) where dimer-sized RsbW would be expected to elute. Perhaps RsbW dimerizes in the absence of RsbV or σ^B. Dimers of the RsbW homolog SpoIIAB have also been reported (12). Unlike RsbW, RsbV appears to exist as a monomer when not associated with RsbW. The monomeric RsbV present in $RsbW^+$ extracts is likely to be modified (see below); however, the modification is unlikely to be the reason for RsbV's failure to dimerize. Unassociated RsbV exits the gel filtration column in essentially the same fractions in $RsbW^-$ extracts (Fig. 1B), where it is not modified, as it does in the $RsbW^+$ extracts (Fig. 1A), where it is altered. Our gel filtration analysis also reinforces the notion that RsbX does not regulate σ^B-dependent transcription by changing the interactions that take place between RsbV and RsbW. We could find no evidence that the presence or absence of RsbX influenced the associations of RsbW under the experimental conditions used in this study (Fig. 1A versus E) or that RsbX itself bound to any of the other products of the *sigB* operon (Fig. 4A and B).

On the basis of an overlap between the *rsbW* and *sigB* genes and an observed reduction in σ^B abundance in a *B. subtilis* strain with a frameshift mutation in *rsbW*, it was suggested that the synthesis of σ^B is translationally coupled to the upstream *rsbW* gene (3, 19). Although this is still likely to be true, the putative coupling between RsbW and σ^B does not appear to produce these proteins in equal molar ratios. The culture conditions under which the *sigB* operon was expressed in this study (induced from P_{SPAC} during exponential growth) does not induce σ^B-dependent transcription (3, 8). As such, very little E-σ^B would be expected to form, and most, if not all, of the σ^B should remain complexed with RsbW. If σ^B and RsbW are synthesized in an equimolar ratio, we would anticipate that most of the RsbW should also be bound to σ^B under such a circumstance. Although, as expected, we detect less than 5% of the σ^B present in these extracts to be excluded from the gel filtration column and presumably bound to RNA polymerase (data not shown), there appears to be sufficient RsbW present in the extracts to not only associate with the remaining σ^B but to also form RsbW-RsbV complexes (Fig. 1A) or RsbW homodimers (Fig. 1C). Apparently, RsbW accumulates in excess to σ^B under the conditions that we used to induce their synthesis. This RsbW excess may ensure that there is sufficient regulator to bind and inactivate σ^B under noninducing conditions.

An additional finding of this study is that RsbV exists in at least two forms that are separable by IEF. Our data argue that the more electronegative of the two prominent RsbV species is likely to be created by a RsbW-dependent phosphorylation reaction that can occur both in vivo and in vitro. RsbV is phosphorylated in vitro in a reaction in which purified RsbW is the only other protein present. Thus, it is unlikely that *B. subtilis* proteins other than RsbW are required for the phosphorylation of RsbV. In addition, when RsbV and RsbW are coexpressed and purified from *E. coli*, more than 75% of the RsbV is recovered as the putative phosphorylated form (11). Although RsbW appears to be sufficient for the phosphorylation of RsbV, we have no data on the efficiency of this reaction or whether additional protein factors normally accelerate it. The modification state of RsbV affects the likelihood that it will be associated with RsbW. IEF of gel filtration fractions containing RsbV-RsbW complexes or RsbV alone demonstrated segregation of the form of RsbV that we assume to be phosphorylated to those fractions containing unbound RsbV and the presumed unphosphorylated form of RsbV to those fractions where RsbV is associated with RsbW.

From the data presented above, a simple model for σ^B regulation could be constructed wherein RsbW alternatively binds to either σ^B or RsbV, thereby inhibiting or allowing the formation of σ^B-containing RNA polymerase. The phosphorylation of RsbV could then be a device to inactivate RsbV and remove it from the regulatory circuit. This would free RsbW to interact with σ^B and silence σ^B-dependent transcription. Presumably, a yet to be identified factor interacts with the RsbV-RsbW complex to influence whether RsbW maintains a stable association with RsbV or initiates the phosphorylation of RsbV. The potential archiving of RsbV in an inactive form by phosphorylation may be the basis for the permanent inhibition of σ^B-dependent transcription that has been observed when *sigB* is induced from P_{SPAC} during exponential growth (3, 8). Under these conditions, large amounts of *sigB* operon products are synthesized at a time when σ^B-dependent transcription is not normally active. By our model, this should favor the phosphorylation and inactivation of RsbV. If, as seems likely, RsbW and RsbV are synthesized in excess of σ^B, any significant loss of RsbV due to its phosphorylation could ensure the presence of sufficient surplus RsbW not only to inhibit the σ^B currently present in the cell but also to inactivate newly synthesized σ^B even if this σ^B is now appearing under conditions that would normally induce σ^B-dependent transcription.

The phosphorylation reaction, observed with RsbV-RsbW, also occurs in the SpoIIAA-SpoIIAB system, in which SpoIIAB has been shown to be a kinase that phosphorylates

SpoIIAA in vitro (1, 22). A model has been offered in which the SpoIIAB-dependent phosphorylation of SpoIIAA activates SpoIIAA to bind SpoIIAB tightly and curtail its ability to inhibit σ^F (22). The notion that it is the phosphorylated form of SpoIIAA that binds SpoIIAB comes from the observation that SpoIIAA purified from *E. coli*, and presumably unmodified, is unable to block SpoIIAB's inhibition of σ^F (22). As discussed above, our gel filtration data argue that if phosphorylation does play a role in regulating RsbV's ability to interact with RsbW, it is the unphosphorylated form of RsbV that likely binds to RsbW to block its inhibition of σ^B. We could find no evidence of complexes between phosphorylated RsbV and RsbW. Moreover, Alper et al. (1) have shown that phosphorylation of SpoIIAA greatly reduces its association with SpoIIAB. Thus, for both the σ^F and σ^B systems, it seems most likely that phosphorylation of the anti-anti-σ factor (SpoIIAA or RsbV) is a mechanism for decreasing σ activity.

Although our results suggest that phosphorylation of RsbV by an RsbW-dependent reaction reduces the association of RsbV with RsbW, we are uncertain of the role of this reaction in the normal regulation of σ^B. We have not yet found an environmental condition under which there is a marked shift in the phosphorylation state of RsbV, even in conditions under which σ^B-dependent genes are induced. It is possible that phosphorylation of RsbV represents a regulatory device for controlling the gross activity of σ^B and that additional mechanisms are used for more subtle changes in its activation state. One such mechanism is suggested by Alper et al., who showed that the ratio of ATP to ADP directly affects the binding of SpoIIAB to either σ^F or SpoIIAA and proposed that a similar adenosine nucleotide switch could control RsbW binding to σ^B or RsbV (1). Alternatively, given that σ^B-dependent transcription appears to be inducible by several different environment stimuli (1, 3, 7, 16), regulation of σ^B by phosphorylation of RsbV may be related only to a subset of these stimuli. It is likely that the mechanism of σ^B activation will prove to involve more factors than we presently know.

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