Opposing pairs of serine protein kinases and phosphatases transmit signals of environmental stress to activate a bacterial transcription factor

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The general stress response of the bacterium *Bacillus subtilis* is governed by a signal transduction network that regulates activity of the σ^B^ transcription factor. We show that this network comprises two partner-switching modules, RsbX-RsbS-RsbT and RsbU-RsbV-RsbW, which contribute to regulating σ^B_. Each module consists of a phosphatase (X or U), an antagonist protein (S or V), and a switch protein/kinase (T or W). In the downstream module, the W anti-σ factor is the primary regulator of σ^B^ activity. If the V antagonist is phosphorylated, the W switch protein binds and inhibits σ^B_. If V is unphosphorylated, it complexes W, freeing σ^B^ to interact with RNA polymerase and promote transcription. The phosphorylation state of V is controlled by opposing kinase (W) and phosphatase (U) activities. The U phosphatase is regulated by the upstream module. The T switch protein directly binds U, stimulating phosphatase activity. The T-U interaction is governed by the phosphorylation state of the S antagonist, controlled by opposing kinase (T) and phosphatase (X) activities. This partner-switching mechanism provides a general regulatory strategy in which linked modules sense and integrate multiple signals by protein–protein interaction.

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How cells sense and integrate multiple environmental and metabolic signals to establish new patterns of gene expression is a fundamental problem in cell biology. The general stress response of the bacterium *Bacillus subtilis* provides a simple system to examine this problem at the molecular level. The general stress response is mediated by σ^B_, an alternative σ factor that binds RNA polymerase core enzyme to confer a new promoter recognition specificity (Haldenwang and Losick 1980). This new holoenzyme form then directs transcription of at least 40 unlinked genes, the products of which are thought to counter a variety of stresses as cells persist in a slowly growing or nongrowing state (Boylan et al. 1993a, b; Völker et al. 1994; Engelmann et al. 1995; Hecker et al. 1996).

The activity of σ^B_ is regulated post-translationally in response to two broad classes of stress: (1) signals of energy stress, such as those imposed by entry into the stationary growth phase, the addition of uncouplers of oxidative phosphorylation to the growth medium, or starvation for carbon, phosphate, or oxygen; and (2) signals of environmental stress, such as osmotic stress, heat shock, or ethanol shock (Benson and Haldenwang 1993b; Boylan et al. 1993a; Alper et al. 1994; Völker et al. 1994; Voelker et al. 1995b). All of these stress signals are conveyed to σ^B_ via a signal transduction pathway encoded by the *rsb* [regulator of sigma B] genes that, together with the structural gene for σ^B_, comprise the *sigB* operon (Benson and Haldenwang 1992; Boylan et al. 1992; Völker et al. 1995a,b, Wise and Price 1995; Kang et al. 1996). The current model for the relationship among the Rsb regulators is shown in Figure 1.

Different components of the σ^B_ signal transduction pathway respond to different stress signals. RsbV and RsbW are required to convey signals of both energy stress and environmental stress [Boylan et al. 1993a; Alper et al. 1994; Voelker et al. 1995b]. The RsbW negative regulator is an anti-σ factor that binds directly to σ^B_ and maintains it in a transcriptionally inactive complex; the RsbV positive regulator is an anti-anti-σ factor that counters the action of RsbW, allowing σ^B_ to direct transcription of its target genes [Benson and Haldenwang 1992, 1993a; Boylan et al. 1992; Dufour and Haldenwang 1994; Alper et al. 1996]. The RsbX, RsbS, RsbT, and RsbU regulators act upstream from the RsbV-RsbW pair and are apparently necessary to transmit signals of environmental stress, but the molecular mechanism underlying this transmission is unknown [Voelker et al. 1995a,b, Wise and Price 1995; Kang et al. 1996]. In con-

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The anti-σ factor mechanism that regulates σB activity is found in at least one other signal transduction pathway in *B. subtilis*. This pathway governs the activity of σB, which controls gene expression in the forespore compartment of the developing sporangium. σB activity is regulated at the post-translational level by the SpoIAB anti-σ factor and the SpoIIA anti-σ factor, which are homologs of the RsB and RsV regulators of σB (Kalman et al. 1995). RsbW has a kinase activity that can modify and inactivate its RsbV antagonist (Dufour and Haldenwang 1993), none of the Rsb proteins form a linear signal transduction pathway that ultimately influences the activity of the RsbW anti-σ factor (Kang et al. 1996). However, the order of action of the RsbX and RsbS regulators, as well as that of the RsbT and RsbU regulators, could not be established solely by genetic means. Here we employ a biochemical approach to demonstrate the activities, order of action, and physical associations among the RsbS, RsbT, RsbU, and RsbX regulators.

According to this model, the RsbW anti-σ factor has a choice of binding partners, either RsbV or σB. In addition to its anti-σ activity, RsbW also possesses a kinase activity directed toward its RsbV antagonist (Dufour and Haldenwang 1994). When cellular energy levels are satisfactory, RsbW phosphorylates RsbV and renders it incapable of binding RsbW, which remains free to bind and inactivate σB. However, when cellular energy levels fall, as upon entry into the stationary growth phase, unphosphorylated RsbV is able to sequester RsbW and thereby free σB (Dufour and Haldenwang 1994; Alper et al. 1996). How might the upstream Rsb regulators influence the phosphorylation state of RsbV in order to convey signals of environmental distress to σB? Here we present biochemical evidence that RsbS and RsbT function by means of a partner-switching mechanism controlled by the phosphorylation state of RsbS. We further demonstrate that the alternate partner to which RsbT binds is the RsbU positive regulator of σB, and that this binding event stimulates an RsbU phosphatase activity specific for RsBP. These findings define the molecular mechanism by which the upstream elements of the σB regulatory network communicate signals of environmental stress to the downstream elements, RsbV and RsbW. Last, we show that the RsbX negative regulator possesses a phosphatase activity specific to RsBP, and suggest that the RsbX phosphatase provides one route by which environmental signals enter the system.

**Results**

A genetic analysis of the σB regulatory network has outlined a multicomponent signal transduction pathway, as shown in Figure 1 (Kang et al. 1996). The complexity of this pathway requires a biochemical approach to determine the roles and physical contacts of the system components. We therefore analyzed their activities and interactions in vitro using purified wild-type and mutant Rsb proteins, in vivo in yeast cells using the two-hybrid system, and in vivo in *B. subtilis* using controlled expression of the RsbT regulator. In the next three sections we demonstrate that RsbS and RsbT function by a partner-switching mechanism controlled by the phosphorylation state of RsbS.

**RsbT possesses a kinase activity specific for RsbS**

The phenotypes caused by substitution of either alanine or aspartate for serine 59 of RsbS, which had opposite regulatory consequences in vivo, suggested that RsbS activity was modulated by a phosphorylation event (Kang et al. 1996). To address this question directly, we separately overexpressed RsbS and RsbT in *E. coli* and puri-
fied these proteins by nickel affinity chromatography, as described in Materials and Methods. As shown in Figure 2A, RsbT possessed a kinase activity that specifically phosphorylated RsbS (lane 4) but not the RsbS homolog, RsbV (lane 7). Moreover, substitution of alanine for serine residue 59 in RsbS abolished its ability to serve as a substrate for RsbT (lane 6). In contrast, as shown in Figure 2B, RsbW possessed a kinase activity that specifically phosphorylated RsbV but not RsbS (lanes 4 and 7), and substitution of alanine for serine residue 56 in RsbV abolished its ability to serve as a substrate for RsbW (lane 6). We conclude that serine residues 59 in RsbS and 56 in RsbV are important for the phosphorylation event in vitro. By analogy to the homologous SpoIIAA–SpoIAB regulatory pair, for which the sole site of phosphorylation was shown to be serine 58 (Najafi et al. 1995), we infer that conserved serines 59 and 56 are also the sites of phosphorylation for RsbS and RsbV, respectively. Consistent with this, biochemical analysis showed that the phosphate group of RsbS-P was base-labile and acid-stable, indicating that the modified residue was either a serine or a threonine (data not shown). From the sum of these results, we conclude that RsbT is a serine protein kinase that phosphorylates RsbS on serine 59. We also confirm and extend previous results (Dufour and Haldenwang 1994; Alper et al. 1996) to conclude that RsbW is a serine kinase that phosphorylates RsbV on serine 56.

**Interaction of RsbS and RsbT**

We demonstrated next that the strength of the RsbS and RsbT interaction is altered by the same substitutions at conserved serine 59 that alter RsbS regulatory function in vivo. The binding of RsbS to RsbT was detected using two different assays: a chemical cross-linker that generated covalent complexes between the two proteins, and a yeast two-hybrid system in which direct protein–protein interactions promoted transcription of a *lacZ* reporter gene (Fields and Song 1989).

In the cross-linker experiments, unlabeled RsbS and [35S]methionine-labeled RsbT were mixed and treated with ethylene glycol-bis succinimidylsuccinate (EGS), which cross-links lysine residues that are about 16.1 Å apart. As shown in Figure 3, incubation of labeled RsbT with cross-linker (lane 2) produced only a single band with the same mobility as untreated RsbT (lane 1). However, when unlabeled RsbS was added to labeled RsbT (lane 3), incubation with cross-linker generated one prominent, high-molecular-mass species of about 30 kDa and one less prominent species of about 44 kDa. Both of these new bands were eliminated in parallel cross-linking experiments when unlabeled RsbV [an RsbS homolog] was added in place of RsbS (lane 4) and were restored when a mixture of unlabeled RsbS and unlabeled RsbV was added (lanes 5–7).

**Figure 2.** RsbT and RsbW have specific kinase activities directed at their antagonist proteins. (A) Purified RsbS or RsbT, or a mixture of both, was incubated with [γ-32P]ATP as described in Materials and Methods and then separated by electrophoresis on a polyacrylamide gel. The positions of RsbS and RsbT proteins were detected by Coomassie blue staining (lane 1) and labeled bands were detected by autoradiography (lanes 2–7). Reaction mixtures contained 200 ng of RsbT (lane 2), 200 ng of RsbV (lane 3), 200 ng of RsbT plus 200 ng of RsbS (lane 4), 200 ng of RsbS plus 5 μl of the coelution fraction of an extract from the RsbT expression strain (PE5371) grown without IPTG induction (lane 5), 200 ng of RsbT plus 200 ng of RsbSS59A (lane 6), and 200 ng of RsbT plus 200 ng of RsbV (lane 7). (B) Purified RsbV or MBP-RsbW, or a mixture of both, was incubated with [γ-32P]ATP as described in Materials and Methods and then separated by electrophoresis on a polyacrylamide gel. Positions of RsbV and MBP–RsbW proteins were detected by Coomassie staining (lane 1) and labeled bands by autoradiography (lanes 2–7). Reaction mixtures contained 600 ng of MBP–RsbW (lane 2), 200 ng of RsbV (lane 3), 600 ng of MBP–RsbW plus 200 ng of RsbV (lane 4), 200 ng of RsbV plus 5 μl of the coelution fraction from MBP–RsbW expression strain (PE5373) without IPTG induction (lane 5), 600 ng of MBP–RsbW plus 200 ng of RsbVS56A (lane 6), and 600 ng of MBP–RsbW plus 200 ng of RsbS (lane 7). Results shown here using the MBP–RsbW fusion were identical to those obtained using purified RsbW protein, supplied by S. Alper, L. Duncan, and R. Losick (not shown). Numbers to the right of each figure indicate positions of the molecular weight standards (given in kilodaltons).

**Figure 3.** RsbT can form a complex with either RsbS or RsbU in vitro. The figure shows an autoradiograph of the products of chemical cross-linking reactions separated by SDS polyacrylamide gel electrophoresis. Reaction mixtures containing [35S]labeled RsbT protein and the unlabeled Rsb proteins specified below were subjected to cross-linking reactions with EGS at 25°C for 40 min, as described in Materials and Methods. Lane 1 contains 35S-labeled RsbT without EGS treatment. In addition to 35S-labeled RsbT, lanes 2–8 contained EGS and the following unlabeled proteins: no other protein (lane 2), 200 ng RsbS (lane 3), 200 ng RsbV (lane 4), 200 ng RsbS and 200 ng RsbV (lane 5), 200 ng RsbU (lane 6), 200 ng RsbX (lane 7), 200 ng RsbU and 200 ng RsbX (lane 8). Positions of molecular weight standards are shown to the right of the figure.
RsbV was added (lane 5). We interpret these new bands as specific complexes of the 13 kDa RsbS and the 14 kDa RsbT, with an apparent stoichiometry of 1:1 in the 30 kDa band.

In the yeast two-hybrid system, the strength of the RsbS-RsbT interaction could be estimated by the degree to which transcription of the lacZ reporter gene was activated by fusions of the \textit{B. subtilis} proteins with the yeast GAL4 DNA binding and activation domains. We measured first the ability of the well-characterized RsbV, RsbW, and \( \sigma^B \) proteins to activate transcription of the yeast reporter gene. The RsbW anti-\( \sigma \) factor and \( \sigma^B \), which are known to interact strongly both in vitro and in \textit{B. subtilis} cells (Benson and Haldenwang 1993a; Dufour and Haldenwang 1994; Alper et al. 1996), also interacted strongly in the yeast two-hybrid system, producing high \( \beta \)-galactosidase activity (Table 1). In contrast, the RsbV anti-anti-\( \sigma \) factor and \( \sigma^B \), which do not interact in vitro or in vivo, showed no detectable interaction in the yeast system.

We then estimated the interactions between the RsbW anti-\( \sigma \) factor and three different forms of the RsbV anti-anti-\( \sigma \) factor. Dufour and Haldenwang (1994) have shown that unphosphorylated RsbV complexes RsbW in \textit{B. subtilis} cells, and phosphorylated RsbV is unable to form this complex. We therefore tested wild-type RsbV and two mutant forms of RsbV in the two-hybrid system. In one RsbV mutant, serine 56 was altered to alanine, which cannot be phosphorylated [see Fig. 2B]. In the other RsbV mutant, serine 56 was altered to aspartate, which is thought to mimic the serine residue in its phosphorylated state (Diederich et al. 1994). In the yeast system, the S56A mutant protein interacted strongly with RsbW, the S56D mutant protein had no detectable interaction, and wild-type RsbV interacted with intermediate strength (Table 1). Therefore, the relative \( \beta \)-galactosidase activities in the yeast two-hybrid system mirrored the known in vivo interactions between these \textit{B. subtilis} regulatory proteins.

We then used the two-hybrid system to demonstrate that RsbS interacted with RsbT and that the S59A and S59D mutant forms of RsbS significantly affected the strength of this interaction. As shown in Table 1, RsbT interacted strongly with the S59A mutant protein yet had no detectable interaction with the S59D mutant protein. These interactions in the two-hybrid system are consistent with the in vivo phenotypes caused by the mutant forms of RsbS (Kang et al. 1996) and support the hypothesis that RsbS and RsbT act by means of a partner-switching mechanism in which the binding choice of RsbT is determined by the phosphorylation state of RsbS.

\textbf{Table 1. Interaction of wild-type or mutant Rsb proteins in the yeast two-hybrid system}

| GAL4 trans-activation | B. subtilis protein fused to GAL4 DNA binding domain$^a$ | \( \sigma^B \) | RsbV | RsbVS56A | RsbVS56D |
|----------------------|--------------------------------------------------------|--------|------|----------|----------|
| RsbW                 | 4498                                                   | 80     | 475  | 12       |
| RsbV                 | 2$^b$                                                  | nd$^c$ | nd   | nd       |

| GAL4 DNA binding$^d$ | B. subtilis protein fused to GAL4 trans-activation domain | RsbU | RsbS | RsbSS59A | RsbSS59D |
|----------------------|------------------------------------------------------------|------|-----|----------|----------|
| RsbT                 | 86$^e$                                                     | 1020 | 3010| 2        |

Values shown are the average \( \beta \)-galactosidase activities (in units per mg protein) of the indicated pairwise comparisons, each determined from four independent double transformants. Intrinsic activity of each single transformant was < 1 unit.

$^a$RsbW manifested intrinsic activation ability when fused with the GAL4 DNA binding domain, so only values for RsbW fused with the GAL4 activation domain are shown. The relative order of the reciprocal comparisons agrees with the order shown here.

$^b$Value for the reciprocal comparison was 1 unit (\( \sigma^B \) fused with the GAL4 activation domain and RsbV with the GAL4 DNA binding domain).

$^c$nd: not determined.

$^d$RsbS and its mutant derivatives had intrinsic activation ability when fused with the GAL4 DNA binding domain, so only values for RsbS fused with the GAL4 activation domain are shown. The relative order of the reciprocal comparisons agrees with the order shown here.

$^e$Value for the reciprocal comparison was 254 units (RsbT fused with the GAL4 activation domain and RsbU with the GAL4 DNA binding domain). By contrast, RsbT displayed no significant interaction with RsbX in either orientation (data not shown).

\textbf{RsbU is the binding partner of RsbT}

If RsbS and RsbT form two of the three components of a partner-switching mechanism, what protein might be the missing partner? We used the two-hybrid system to screen the known Rsb regulators and found that only RsbU interacted with RsbT (Table 1). This finding is consistent with the genetic data that suggests that RsbT acts upstream from RsbU in a linear signal transduction pathway (Kang et al. 1996). To test the in vitro interaction between RsbT and RsbU, we employed a cross-linking experiment similar to that used to detect the interaction between RsbS and RsbT. As shown in Fig. 3, when labeled RsbT was mixed with unlabeled RsbU and treated with EGS (lane 6), the reaction generated two high-molecular-mass species, one of ~60 kDa, the other of ~90 kDa. These new species were absent in a parallel cross-linking experiment in which unlabeled RsbX (an RsbU homolog) was added to labeled RsbT (lane 7) and were restored when a mixture of RsbU and RsbX was added (lane 8). We interpret the appearance of the 60-kDa band as a specific complex of the 39-kDa RsbU and the 14-kDa RsbT, with a presumed 1:1 stoichiometry.

The finding of a direct interaction between RsbT and RsbU in vitro reinforces the interpretation that RsbS, RsbT, and RsbU function by means of a partner-switching mechanism that depends on the phosphorylation state of RsbS:

\[
\text{RsbS-P + RsbT \cdot RsbU \rightleftharpoons RsbS \cdot RsbT + RsbU}
\]
By what mechanism does this partner switch convey signals of environmental stress to the downstream members of the $\sigma^B$ signal transduction pathway, RsbV and RsbW? In the following two sections, we show first that RsbU possesses a phosphatase activity specific for RsbV-P, and second that RsbT significantly stimulates this specific phosphatase activity.

**RsbU has a phosphatase activity specific for RsbV-P, and RsbX has a phosphatase activity specific for RsbS-P**

The finding that RsbU and RsbX each share sequence similarity with the SpoIIE phosphatase of the $\sigma^F$ signaling pathway (Duncan et al. 1995) implied that these Rsb regulators could also bear phosphatase activities. From the order of Rsb action suggested by the genetic analysis (see Fig. 1), we hypothesized that RsbV-P was the target of the presumed phosphatase activity of RsbU, and that RsbS-P was the target of RsbX.

To test this hypothesis, purified RsbV and RsbS were labeled with $[\gamma-32P]ATP$ and used as substrates in phosphatase assays (see Materials and Methods). As shown in Figure 4A, RsbU specifically removed the labeled phosphate from RsbV-P (lanes 1–5) but not from RsbS-P (lanes 6 and 7). As shown in Figure 4B, this phosphate removal had no detectable effect on the level of RsbV protein, excluding the possibility that RsbU might function as a protease rather than as a phosphatase. Notably, similar experiments demonstrated that RsbX had a phosphatase activity specific for RsbS-P and not RsbV-P (Fig. 4C, D). In addition to this difference in substrate specificity, another important difference was that at similar molarities of enzyme and substrate, the RsbX phosphatase was significantly more active than RsbU. Therefore, instead of the incubation temperature of 25°C used for the RsbU reactions (Fig. 4A, B), the RsbX reactions (Fig. 4C, D) were incubated at 4°C. We demonstrate in the following section that activity of the RsbU serine phosphatase could be stimulated materially by the addition of the RsbT positive activator.

**RsbT stimulates the RsbU phosphatase activity**

Because RsbT interacts directly with RsbU in vitro (Fig. 3), modulation of the RsbU phosphatase activity by direct protein–protein interaction offers an attractive explanation of how the upstream elements of the Rsb network could communicate stress signals to the downstream RsbV and RsbW regulators. If this were the case, we would predict that RsbT would stimulate RsbU phosphatase activity in vitro, and that the controlled expression of RsbT would stimulate $\sigma^B$ activity in vivo in an RsbU-dependent manner. With regard to stimulation of RsbU activity in vitro, when a rate-limiting amount of purified RsbU protein was included in reaction mixtures with $32P$-labeled RsbV-P substrate, phosphatase activity clearly was stimulated by the addition of increasing amounts of purified RsbT protein (Fig. 5A, B, lanes 1–5). This stimulation was dependent on the presence of RsbU (lane 6). By contrast, in reactions containing the highest amount of added RsbT, RsbU did not remove the label from RsbS-P (lanes 7 and 8). We conclude that the addition of RsbT stimulates the phosphatase activity of RsbU but does not alter the specificity of the reaction. The activity of RsbU against RsbV-P was also enhanced.
A similar experiment was conducted to investigate the effect of RsbT and manganese on the activity of the RsbX phosphatase. As shown in Figure 5C,D, when a limiting amount of purified RsbX protein was included in reaction mixtures with 32P-labeled RsbS-P substrate, phosphatase activity did not respond to added RsbT [lanes 1–5], nor did added RsbT change the specificity of the reaction [lanes 7 and 8]. However, RsbX activity was enhanced by added manganese [lane 9]. This enhancement was dependent on RsbX [lane 10] and did not alter substrate specificity [lane 11]. Thus, the activities of the RsbU and RsbX serine phosphatases—as well as that of the related SpoIIE phosphatase (Duncan et al. 1995)—were both enhanced by manganese, which is a cofactor for a number of other serine phosphatases (Barton et al. 1994). But only the RsbU phosphatase activity was stimulated by RsbT in vitro.

In eukaryotic signal transduction pathways, the activities of serine-threonine phosphatases are often regulated via modification events catalyzed by specific protein kinases (Hunter 1995). Because RsbT possesses a protein kinase activity directed toward RsbS (Fig. 2), we tested whether the kinase activity of RsbT could also modify the RsbU protein in vitro and thereby control its phosphatase activity. Under the same reaction conditions in which RsbT readily phosphorylated RsbS [Fig. 2], RsbT did not phosphorylate RsbU [not shown]. We therefore conclude that the most likely mechanism by which RsbT stimulates RsbU phosphatase activity in vitro is the direct protein–protein interaction demonstrated in Figure 3 and Table 1.

With regard to stimulation of RsbU activity in vivo, we first engineered a multicopy plasmid to express RsbT under control of the lacI repressible-IPTG inducible promoter Pspac. This plasmid was transformed into two different B. subtilis strains, one carrying a wild-type sigB operon and the other a null mutation within the RsbU coding region. To provide an assay for σB activity, each strain also carried a single-copy transcriptional fusion between the well-characterized σB-dependent ctc promoter and a lacZ reporter gene (Moran et al. 1982; Igo et al. 1987; Boyle et al. 1992). Both strains were grown in rich medium and IPTG was added in early exponential growth to induce expression of the multicopy rsbT gene. In the absence of stress, σB is normally silent during exponential growth (Boylan et al. 1992, 1993; Benson et al. 1993b; Voelker et al. 1995b). However, as shown in Figure 6A, σB activity was strongly induced by controlled expression of rsbT in the strain bearing the wild-type sigB operon. In contrast, σB activity was not induced significantly by expression of rsbT in the strain harboring the rsbU null allele. As shown in Figure 6B, both the σB regulatory network and the reporter fusion in these strains were capable of responding to the stress of entry into stationary phase, which is independent of RsbU function. We conclude that increased expression of rsbT alone is sufficient to activate σB in exponentially growing cells, and that this activation process requires RsbU function. A model of σB regulation based on the sum of our results is shown in Figure 7.

by manganese addition [lane 9], this enhancement was dependent on RsbU [lane 10] and did not alter substrate specificity [lane 11].
Figure 6. Activation of σB by RsbT requires RsbU function in vivo. [A] Effect of rsbT overexpression on β-galactosidase activity of a σB-dependent ctc-lacZ transcriptional fusion. Cells were grown in buffered Luria broth medium (Boylan et al. 1993a) to early exponential phase. At time 0, IPTG (1 mM final concentration) was added to each culture to induce expression of the rsbT gene on the multicopy plasmid pCK35. Samples were taken and assayed for β-galactosidase activity. (A,B) Activity of strain PB510 [rsbUA::ermC amyE::ctc-lacZ trpC2 pCK35]. [C] activity of strain PB548 [amyE::ctc-lacZ trpC2 pCK35]. [B] Effect of entry into stationary phase on β-galactosidase activity. The two strains tested in A were sampled and assayed for β-galactosidase activity. Growth of PB548 was essentially the same.

Discussion

Reversible covalent modification of proteins is a universal mechanism for signal transduction in both prokaryotic and eukaryotic cells. In bacteria, signaling proteins are often phosphorylated, a modification that principally appears to influence their conformation and, therefore, their binding contacts. These binding contacts can be with another protein, between their own domains and subunits, or with a specific nucleic-acid sequence. The so-called two-component systems, with their conserved transmitter and receiver domains, are the most extensively studied signal transduction paradigms in bacteria [Parkinson 1993].

Here we have presented evidence indicating a more widespread role for a different signaling paradigm in bacteria, the partner-switching mechanism originally shown to negatively regulate the σB and σF transcription factors in B. subtilis [Alper et al. 1994, 1996; Diederich et al 1994; Dufour and Haldenwang 1994]. In this regulatory strategy, activity of an anti-σ factor is controlled by direct protein–protein interaction with a specific antagonist protein, an anti-anti-σ factor that maintains the anti-σ factor in an inactive complex. Once phosphorylated, the anti-anti-σ factor cannot form a complex with the anti-σ factor, which can then directly interact with the target σ to inhibit its activity.

In the case of the RsbT activator of the RsbU phosphatase, we have now extended the partner-switching paradigm to encompass activation as well as inhibition, and to target at least one protein with an enzymatic activity different from the transcription factors for which the mechanism was originally described. As is the case for the RsbW and SpoIAB anti-σ factors, the RsbT phosphatase activator appears to be controlled by direct protein–protein interaction with a specific antagonist protein. According to the model shown in Figure 7, upon phosphorylation of the RsbS antagonist protein, RsbT is released to activate the RsbU phosphatase, which then specifically removes the serine phosphate from RsbV-P. This activation of RsbU by RsbT is also apparently mediated by direct protein–protein interaction. RsbU is a member of a phosphatase family that includes RsbX in the σB regulatory network (Fig. 4) and SpoIIIE in the σF regulatory network [Duncan et al. 1995]. The region of identity shared among the three phosphatases lies within their carboxy-terminal portions, suggesting that this region contains the phosphatase domain. Because RsbU possesses an extended amino-terminal region that RsbX lacks [Wise and Price 1995], and because RsbT binds RsbU and not RsbX (Fig. 3 and Table 1), we suggest that the amino-terminal extension of RsbU provides at least part of the region of contact. In this view, the amino-terminal extension of RsbU could inhibit phosphatase activity until RsbT binds to alter RsbU conformation, thereby exposing the phosphatase domain. Consistent with this notion, the amino- but not the carboxy-terminal region of RsbU is sufficient to interact with RsbT in the yeast two-hybrid system [C.M. Kang, K. Vija, and C.W. Price, unpubl.].

What is the role of the RsbX phosphatase in the σB regulatory pathway? RsbS is a central component of the upstream activation network, and we propose that the phosphorylation state of RsbS governs whether the RsbT activator engages the RsbU phosphatase. Because RsbS-P...
is the target of the RsbX phosphatase activity, the level and activity of RsbX determine at least in part the phosphorylation state of RsbS. When RsbX dephosphorylates RsbS-P, RsbS binds and inactivates RsbT, reducing the positive regulatory input that is channeled via RsbU from the upstream to the downstream regulators [see Fig. 7]. Thus RsbX activity is inversely related to σB activity.

We therefore envision two possible regulatory roles for the RsbX phosphatase. First, its activity could be controlled negatively by environmental signals to increase σB activity in response to stress, and this might provide one route by which external signals enter the network. Second, we deduce from the DNA sequence of the operon that expression of sigB and rsbX might be coupled translationally [Kalman et al. 1990]. If this were the case, then the molar concentration of RsbX could afford an indirect measure of the molar concentration of σB. We might then imagine that RsbX provides a mechanism to regulate the steady-state level of σB in cells that are not yet subjected to stress. That is, when σB and RsbX concentrations rise above a target level, σB activity would be reduced appropriately by action of the multicomponent regulatory network, leading to reduced expression of the sigB and rsbX structural genes from the σB-dependent promoter of the sigB operon (see Fig. 1). In contrast, when σB and RsbX concentrations fall below the target level, σB activity would be increased, restoring the optimum steady-state level of σB to ensure a rapid, autocatalytic stress response. These postulated roles for RsbX are not mutually exclusive.

Based on the results presented here, we suggest that the partner-switching mechanism is a more general regulatory strategy than previously supposed. The partner-switching components share no obvious homology with bacterial two-component systems. In the two-component systems, the transmitter is a histidine protein kinase that autophosphorylates a conserved histidine residue before transferring the phosphate group to a conserved aspartate on the response regulator (Parkinson 1993). Many of the histidine protein kinases also possess autophosphorylation activity, and the phosphatase activity of the histidine protein kinases, retaining only the ATP binding motif. Furthermore, the target antagonist proteins of the partner-switching kinases are phosphorylated on a serine rather than a aspartate residue and bear no resemblance to the receiver domain of two-component response regulators. However, like the two-component systems, the molecular mechanism by which the partner-switching systems convey signals is by reversible phosphorylation events that control protein contacts. And like the two-component systems, the partner-switching systems also appear to consist of modular components that can be arranged in a variety of configurations to accomplish different signaling tasks.

In the case of the σB regulatory network, the complexity of the linear signal transduction pathway may be required to sense and integrate the multiple signals that control the activity of a single transcription factor. In addition to providing a number of discrete steps at which signal transduction can be regulated, the two halves of the regulatory network carry different kinds of signals. The downstream members of the pathway—RsbV and RsbW—are thought to sense and convey signals of energy stress, whereas the upstream members—RsbX, RsbS, RsbT, and RsbU—are thought to convey signals of environmental stress [Alper et al. 1994; Voelker et al. 1995b; Kang et al. 1996]. Notably, with the exception of RsbX, the order of action of the Rsb regulators is the same as their order in the operon. Given the obvious homologies among the Rsb regulators [Fig. 1], we speculate that the two halves of the signal transduction pathway arose by a tandem duplication that produced two signaling modules, each arranged in the order of phosphatase–antagonist–switch protein. Each module would then be free to elaborate the ability to sense and transmit different signals, and the multiple-module configuration would provide the means to integrate these different signals with opposing protein kinase and protein phosphatase activities.

We note that these partner-switching modules provide a formally similar integrative function to the phosphate-requiring circuitry that controls initiation of the sporulation process in *B. subtilis*. The phosphate-requiring circuitry is an elaboration of the two-component mechanism that permits the cell to integrate nutritional, cell-density, and cell-cycle signals by means of opposing protein kinase and protein phosphatase activities [Burbulys et al. 1991; Ireton et al. 1993; Perego et al. 1994, 1996; Perego and Hoch 1996]. Thus two different signal transduction pathways, each based upon a different mechanism, have adopted similar strategies to address the common problem of sensing and integrating multiple signals.

Given the biochemical activities and protein–protein interactions described here, we would not expect additional components to be interposed between the Rsb regulators shown in Fig. 7. Of course, accessory proteins that modify either the phosphorylation and dephosphorylation activities of network components or the contacts among them may remain to be discovered, and the question of how environmental signals enter the upstream half of the pathway remains to be addressed.

Materials and methods

**Bacterial strains and genetic methods**

*B. subtilis* strains are derivatives of the 168 Marburg strain PB2. Recombinant DNA methods and *B. subtilis* transformations were as previously described [Kang et al. 1996];

**Construction of bacterial plasmids for Rsb protein expression**

Genes for RsbS, RsbT, RsbU, RsbV, and RsbX proteins were cloned into the PET15b expression vector [Novagen, Madison, WI]. PET15b encodes a six-histidine tag and a thrombin cleavage site, placing the fusion construction under control of a T7 promoter. Wild-type reading frames were amplified by PCR using...
genomic DNA of strain PB2 as template. The rsbS frame containing the S59A substitution and the rsbV frame containing S56A were amplified from pCK5 [Kang et al. 1996] and pCK7 [C.M. Kang and C.W. Price, unpubl.], respectively.

The pMAL-p2 vector [New England Biolabs, Beverly, MA] was used to make a fusion between the E. coli maltose binding protein (MBP) and the rsbW reading frame. For the pMAL-p2 fusion as well as for each of the pET15b fusions, DNA sequencing confirmed that the fusion nexus was as predicted and that no mutations had been introduced by PCR amplification.

Purification of Rsb proteins

The seven Rsb proteins in the pET15b vector were purified from E. coli BL21(DE3)/plysS under native conditions on a His-Tag column [Novagen] according to the manufacturer’s protocol, with the exception that lower imidazole concentrations were used for elutions: 100 mM for RsbU, RsbV, RsbVS56A, and RsbX, 200 mM for RsbS, RsbS59A, and RsbT. Additionally, Triton X-100 (0.05% final concentration) was included in the loading and elution buffers for RsbX. The MBP-RsbW fusion was purified on an amylose column [New England Biolabs] according to the manufacturer’s protocol. All proteins were judged to be >95% pure by Coomassie staining [not shown].

To provide negative control fractions for the phosphorylation and dephosphorylation assays, for each protein purified, we also ran a parallel purification of the uninduced, fusion-bearing strain. Purified Rsb proteins were desalted and concentrated using an Amicon Microcon-3 ultrafiltration apparatus, then resuspended in either storage buffer [10 mM Tris (pH 8.0), 50 mM NaCl, 1 mM DTT, 50% glycerol] or Xlink-storage buffer [in which 20 mM HEPES (pH 7.5) replaced the Tris of storage buffer]. Suspensions were kept at 4°C, reactions were stopped by adding 5 μl of 5 × sample loading buffer, then separated on SDS polyacrylamide gels. Protein bands were stained with Coomassie blue and phosphorylated proteins were detected by autoradiography.

Chemical cross-linking reactions

Cross-linking was carried out in 30 μl reaction mixtures containing 1 μl of 35S-labeled E. coli cell extract with RsbS-T [1–2 μg total protein], 20 mM HEPES [pH 7.5], 150 mM NaCl, 10 mM MgCl2, and 1 mM DTT. Each mixture further contained either no protein (negative control) or 200 ng each of the unlabeled Rsb proteins indicated in the legend to Figure 3. After incubation at 0°C for 15 min, EGS was added to 2 mM final concentration. Reactions were continued for 40 min at 25°C, then terminated by adding lysine to 50 mM final concentration. Following addition of 7.5 μl of 5× sample loading buffer, samples were heated at 85°C for 5 min, separated on an SDS polyacrylamide gel, and assayed by autoradiography.

Construction and use of GAL4 fusions in the yeast two-hybrid system

We used the Matchmaker Two-Hybrid System [Clontech, Palo Alto, CA] as an assay for protein–protein interaction among Rsb regulators. Genes for RsbS, RsbT, RsbU, RsbV, RsbW, RsbX, and α proteins were cloned into pGBT9 for fusions to the yeast GAL4 DNA binding domain and into pGAD424 for fusions to the yeast GAL4 activation domain. Wild-type reading frames were amplified by PCR using genomic DNA from strain PB2 as template; the rsbS frames containing the S59A and S59D substitutions were amplified from pCK5 and pCK6 [Kang et al. 1996], and the rsbV frames containing the S56A and S56D substitutions were amplified from pCK7 and pCK8 [C.M. Kang and C.W. Price, unpubl.], respectively.

DNA sequence analysis of each of the constructions verified the expected fusion junction and the absence of PCR-generated mutations. To test the interactions among Rsb regulators, a pGAD424 construction carrying one Rsb protein fused to the GAL4 activating domain was cotransformed into the Saccharomyces cerevisiae SFY526 host strain together with a pGBT9 construction carrying another Rsb protein fused to the GAL4 DNA binding domain. Double transformants were selected on minimal medium; four independent transformants for each combination of plasmids were purified for further use. Each independent transformant was grown in minimal medium, harvested during logarithmic growth, then assayed for β-galactosidase activity according to Miller [1972], except that yeast extracts were prepared by freezing cell suspensions in liquid nitrogen and thawing at 37°C. Protein levels were determined using the Protein Assay Reagent [Bio-Rad Laboratories, Richmond, CA]; specific activity was defined as ΔA420× 1000/min per mg protein.

In vitro phosphorylation and dephosphorylation assays

In vitro phosphorylation assays were performed as described by Min et al. [1993], except that the reactions were done in a 20-μl volume containing 20 μM unlabeled ATP and 5 μCi of [γ-32P]ATP. The amounts of purified Rsb protein added are indicated in the legend to Figure 2. Reactions were terminated with 5 μl of 5 × sample loading buffer. Samples were heated at 85°C for 5 min and separated on SDS polyacrylamide gels. Next, protein bands were stained with Coomassie blue and phosphorylated proteins detected by autoradiography.

For dephosphorylation assays, the RsbT and RsbW kinases were used to make the RsbS-P and RsbV-P substrates, respectively. For the RsbS-P substrate, the His tag was removed from the purified His–RsbS protein by thrombin digestion. Next, 20 μg of RsbS protein [without His tag] was phosphorylated by 2 μg of His–RsbT in a 200-μl reaction volume, using the same protocol specified for the phosphorylation assay. His–RsbT was then removed by batch precipitation with His Bind Resin [Novagen]. Similarly, for the RsbV-P substrate, 20 μg of His–RsbV protein was phosphorylated using 2 μg MBP–RsbW, which was removed by batch precipitation with amylose resin [New England Biolabs]. Last, free nucleotides were removed from the RsbS-P and RsbV-P substrates by washing twice with 1 mM NaCl on Amicon Microcon-3 filters.

Dephosphorylation reactions were done in 20-μl reaction volumes containing 50 mM Tris (pH 8.0), 100 mM NaCl, 10 mM MgCl2, and 1 mM DTT, together with the protein indicated in the legends to Figures 4 and 5. After 30 min at either 25°C or 4°C, reactions were stopped by adding 5 μl of 5 × sample loading buffer. Samples were heated at 85°C for 5 min, then separated by SDS–PAGE. Unlabeled protein bands were visualized with Coomassie blue and phosphorylated proteins were detected by autoradiography.
Overexpression of the rsbT product in B. subtilis

We placed the rsbT reading frame under control of the inducible Pspac promoter of the multicopy expression vector pDG148 (Stragier et al. 1988). Based on the DNA sequence, expression of rsbT may be coupled translationally to expression of the upstream rsbR and rsbS genes. Consequently, we used PCR to amplify the fragment of interest from pSA50, which carries an in-frame deletion that removes most of the rsbR and rsbS coding regions (S. Akbar and C.W. Price, unpubl.). The resulting plasmid, pCK35, carries the ribosomal binding site, the first nine codons of rsbR fused to the last 14 codons of rsbS, and the entire rsbT coding region, all under Pspac control. pCK35 was transformed into B. subtilis PB198 [wild-type] and PB244 (rsbUΔ1::ermC). A α3-dependent ctc-lacZ fusion in single copy at the amyE locus (Moran et al. 1982; Igo et al. 1987, Boylan et al. 1992) reported α3 activity in these strains. β-galactosidase was assayed according to Miller (1972), as described (Kang et al. 1992) reported αβ activity in these strains.

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