RsbU is a positive regulator of the activity of $\sigma^B$, the general stress-response $\sigma$ factor of Gram-$+$ microorganisms. The N-terminal domain of this protein has no significant sequence homology with proteins of known function, whereas the C-terminal domain is similar to the catalytic domains of PP2C-type phosphatases. The phosphatase activity of RsbU is stimulated greatly during the response to stress by associating with a kinase, RsbT. This association leads to the induction of $\sigma^B$ activity. Here we present data on the activation process and demonstrate in vivo that truncations in the N-terminal region of RsbU are deleterious for the activation of RsbU. This conclusion is supported by comparisons of the phosphatase activities of full-length and a truncated form of RsbU in vitro. Our determination of the crystal structure of the N-terminal domain of RsbU from Bacillus subtilis reveals structural similarities to the regulatory domains from ubiquitous protein phosphatases and a conserved domain of $\sigma$-factors, illuminating the activation processes of phosphatases and the evolution of “partner switching.” Finally, the molecular basis of kinase recruitment by the RsbU phosphatase is discussed by comparing RsbU sequences from bacteria that either possess or lack RsbT.

Reversible phosphorylation of proteins is the predominant regulatory mechanism in biology, modulating cellular processes such as signaling, division, and development. The phosphorylation of regulatory proteins by protein kinases effects a change in their function and structure (1), reversed by the action of protein phosphatases, which restore the regulatory proteins to their original, unphosphorylated state. Hence the cellular response is determined by controlling the enzymatic activities of the mutually antagonistic kinases and phosphatases. Protein phosphatases can be divided into three major groups, defined by their substrate specificity (2): phosphotyrosine (further subdivided into Cdc25 and the low molecular weight phosphotyrosine phosphatases), phosphoserine/threonine (further subdivided into protein phosphatase P and M families), and phosphoaspartate phosphatases (e.g. Rap and Spo0E from Bacillus). In addition, there is the dual-specific phosphatase group, which can dephosphorylate phosphotyrosine and phosphoserine/threonine substrates, and it has been recorded that several histidine kinases also have phosphohistidine phosphatase activity (3).

In Bacillus subtilis, five serine/threonine protein phosphatases have been identified that belong to the PP2C subgroup of the protein phosphatase M family, namely PrpC (4), SpoIIE (5), RsbP (6), RsbX and RsbU (7). PrpC plays an important regulatory role in stationary phase (8), and SpoIIE regulates differentiation in Bacillus, a process known as sporulation, by forming a complex with the cell division protein FtsZ (5, 9). RsbX, RsbP, and RsbU are involved in the regulation of the alternative $\sigma$ factor, $\sigma^B$, which controls the general stress response of B. subtilis and other Gram$+$ microorganisms, such as the human pathogens Listeria monocytogenes and Staphylococcus aureus (10, 11). Depending on the nature of the stress, the signal is conveyed to $\sigma^B$ by two separate pathways, which converge on phosphorylated RsbV (RsbV-P), the common substrate for RsbP and RsbU (Fig. 1). A decrease in the intracellular energy level activates RsbP, via RapQ (12), whereas environmental stresses such as heat or salt shock, or ethanol treatment, activate RsbU, via the kinase RsbT (7, 13, 14). D Dephosphorylated RsbV subsequently liberates $\sigma^B$ from the transcriptionally inactive $\sigma^B$-RsbW complex, by competing with $\sigma^B$ for binding surfaces on RsbW (15, 16), freeing $\sigma^B$ to bind to core RNA polymerase to activate transcription of the $\sigma^B$ regulon. The alternative binding of RsbW to $\sigma^B$ or RsbV is a regulatory mechanism called “partner switching” (17).

RsbT is related to the anti-$\sigma$ factors RsbW and SpoIIB; all are members of the GHKL family of kinases/ATPases, a group that includes two-component histidine kinases, topoisomerase-

The abbreviations used are: RsbV-P, phosphorylated RsbV; IPTG, isopropyl-$\beta$-D-galactopyranoside; TPR, tetratricopeptide repeat; PP5, protein phosphatase 5.
ases, and chaperones. However, RsbT does not bind, as in the other partner-switching mechanisms, to $\sigma^B$ or anti-$\sigma^B$ factors. During exponential growth RsbT is thought to be sequestered in a large supramolecular complex composed of RsbR and RsbS (18). However, during environmental stress RsbT is liberated from the supramolecular complex after phosphorylating its substrates, RsbR and RsbS. RsbT is then free to associate with, and activate, RsbU (7). RsbU is not a substrate for the kinase activity of RsbT (19), and the precise mechanism of the activation of RsbU by RsbT remains unknown. Analysis of the sequence of RsbU shows that it is composed of two domains, a C-terminal domain of 200 amino acids with sequence homology to other PP2C-type phosphatases and a N-terminal domain of 110 amino acids with no significant homology to any non-RsbU sequences. The simplest hypothesis regarding the role of the N-terminal domain of RsbU is that it exerts an inhibitory influence on the C-terminal, catalytic domain, which is relieved by the binding of RsbT to RsbU.

We report here an investigation into the activation process of RsbU that integrates genetics and molecular and structural biology. The phenotype of *B. subtilis* strains containing deletions in the N-terminal domain of RsbU support the view that this domain plays a critical role in the activation of the phosphatase by RsbT. These results are discussed in the light of our determination of the crystal structure at 1.6-Å resolution of the N-terminal domain of RsbU, and conclusions are drawn as to the nature of the activation process of RsbU.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Media, and Growth Conditions**—The experiments conducted in this study (Table I) were performed with derivatives of *B. subtilis* wild type strain 168 (20). For the construction of the various truncated versions of RsbU, there were two approaches. First, the *rsbU* gene, and truncated versions thereof, were amplified by PCR with chromosomal DNA of *B. subtilis* strain 168 as the template using appropriate primer pairs and a proofreading Taq polymerase. Subsequently, all these PCR products were digested with HindIII and SalI and ligated into the non-integrative plasmid pDG148 (21), which had also been digested with the same enzymes. The different *rsbU* alleles were thus placed under the control of the IPTG-regulated promoter $P_{spac}$. After transformation into *Escherichia coli* strains TOP10 or TG2, and confirmation of the correct DNA sequence of the *rsbU* inserts by sequencing, plasmids carrying the wild type *rsbU* gene, and truncated versions thereof, were amplified by PCR with chromosomal DNA of *B. subtilis* strain 168 as the template using appropriate primer pairs and a proofreading Taq polymerase. Subsequently, all these PCR products were digested with HindIII and SalI and ligated into the non-integrative plasmid pDG148 (21), which had also been digested with the same enzymes. The different *rsbU* alleles were thus placed under the control of the IPTG-regulated promoter $P_{spac}$. After transformation into *Escherichia coli* strains TOP10 or TG2, and confirmation of the correct DNA sequence of the *rsbU* inserts by sequencing, plasmids carrying the wild type *rsbU* sequence (pGK01) and the truncations $\Delta rsbU^2-19$ (pMB21), $\Delta rsbU^3-38$ (pGK02), $\Delta rsbU^5-77$ (pGK03), $\Delta rsbU^5-93$ (pGK04), and $\Delta rsbU^5-134$ (pGK05) were selected for transformation into *B. subtilis* strain BSA140 (Table I). The *B. subtilis* strain BSA140 carries a ctc:: lacZ transcriptional reporter gene fusion, but lacks a functional copy of *rsbU* because of a deletion of an NdeI fragment internal to the *rsbU* structural gene. Transformants...
were selected for their resistance to kanamycin (20 μg ml⁻¹) creating strains BSG14, BSG15, BSG16, BSG17, BSG18, and BSG19, respectively (Table I).

Bacteria were routinely grown under vigorous agitation in a minimal medium described previously (22) supplemented with 0.2% (w/v) glucose as a carbon source and t-cytophanol (0.78 μM). The cultures were inoculated from overnight cultures propagated in minimal media containing kanamycin to an optical density at 540 nm of 0.05. The expression of the plasmid-encoded rsbU variants was induced by the addition of IPTG to a final concentration of 1 mM. Ethanol stress was imposed on the cells during exponential growth phase (A₅₄₀ ≈ 0.3) by the addition of ethanol to a final concentration of 4% (v/v).

In the second approach, B. subtilis strain PB291, previously deleted for rsbU, was transformed with derivatives of plasmid pMLK (23), which directs integration at the amyE locus. A fragment of DNA corresponding to P₆-rsbR-rsbS-rsbT-rsbU was amplified separately for P₆-rsbR-rsbS-rsbT-rsbU (where P₆ represents the α-dependent promoter region of the operon) was excised from plasmid pAW70 (24) by BamHI restriction digest and cloned in pMLK using the BamHI site in amyE. For the cloning of rsbR, rsbS, and rsbT, and a fragment of rsbU coding for the C-terminal catalytic domain only (ΔN-rsbU), the regions corresponding to P₆-rsbR-rsbS-rsbT and ΔN-rsbU were amplified separately for PCR using primers that allow overlaps between the 5’ and 3’ ends, respectively. The annealed PCR products were subsequently used as the template for a further round of PCR to yield a DNA fragment of P₆-rsbR-rsbS-rsbT-ΔN-rsbU, which was also cloned in pMLK at the BamHI site in amyE. Transformants of these strains, which are diploid for rsbR, rsbS, and rsbT, were selected by loss of amylase activity on starch plates. Bacteria were grown in buffered LB, and subjected to 4% (v/v) ethanol to a final concentration of 1 mM. Ethanol stress was imposed on the cells during exponential growth phase (A₅₄₀ ≈ 0.3) by the addition of ethanol to a final concentration of 4% (v/v).

In both cases, the β-galactosidase activity of the ctc::lacZ reporter gene fusion was determined by harvesting 1-ml aliquots of cells at appropriate time points by centrifugation at 4 °C. β-Galactosidase enzyme assays were conducted as previously described (14, 25).

Purification of Full-length RsbT and Truncated Proteins—RsbT was overexpressed in E. coli (BL21) and was purified using a procedure slightly modified from that previously published (18). Cells were disrupted by sonication in 30 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM MgCl₂, 1 mM MnCl₂, and 1 mM dithiothreitol at 30 °C with 30 μM RsbV-P, 0.5 μM RsbU (or C-RsbU) and, unless specified, 1 μM RsbT. The rates of dephosphorylation of RsbV-P were measured at various time intervals by removing 20-μl samples, stopping the reaction in each by the addition of 10 μl of loading buffer (40% glycerol, 200 mM EDTA, and 0.1% bromphenol blue) and placing the sample on ice until analysis by native gel electrophoresis and Coomassie Blue staining. RsbV and RsbP bands are easily separated on a 12% acrylamide gel (16). Eight time points were normally taken per reaction. The gels were scanned and intensities of the bands corresponding to the appearance of RsbV were measured with Scion Image software. The values were then compared with a standard curve of known concentrations of RsbV treated under the same electrophoretic conditions.

Crystallographic Methods—The structure of N-RsbU was determined by selenomethionine MAD phasing. To prepare selenomethionyl-labeled N-RsbU, the E. coli methionine auxotroph, B834 (DE3), was transformed with pETNRsbU, a pET15b derivative that directs the

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**Table I**

| Strain or plasmid | Relevant genotype or features of plasmid | Construction or Ref. |
|-------------------|----------------------------------------|----------------------|
| B. subtilis       |                                        |                      |
| BSA46             | PY22 SPβ ctc::lacZ erm cat 86°         |                      |
| BSA40             | PY22 rbsLΔNΔdel rsbX::pW25 SPβ ctc::lacZ erm cat 86° speβ | This study |
| BG14              | PY22 rbsLΔNΔdel rsbX::pW25 gPK01 SPβ ctc::lacZ erm cat 86° speβ | This study |
| BG15              | PY22 rbsLΔNΔdel rsbX::pW25 gPK02 SPβ ctc::lacZ erm cat 86° speβ | This study |
| BG16              | PY22 rbsLΔNΔdel rsbX::pW25 gPK03 SPβ ctc::lacZ erm cat 86° speβ | This study |
| BG17              | PY22 rbsLΔNΔdel rsbX::pW25 gPK04 SPβ ctc::lacZ erm cat 86° speβ | This study |
| BG18              | PY22 rbsLΔNΔdel rsbX::pW25 gPK05 SPβ ctc::lacZ erm cat 86° speβ | This study |
| BG19              | PY22 rbsLΔNΔdel rsbX::pW25 pMB21 SPβ ctc::lacZ erm cat 86° speβ | This study |
| PB291             | PB2 ΔusrB::ermC SPβ::lacZ trpC2         | 24                   |
| E. coli           |                                        |                      |
| BL21(DE3)         | F’ ompT hsdSMB-125 (F- mB) gal dcm (DE3) | Novagen |
| BS834(DE3)        | F’ ompT hsdSMB-125 (F- mB) gal dcm met (DE3) | Novagen |
| TOP10             | F’ ncrA Δ (mrr-hsdRMS-mlcVC6) 860lacZAM153lacX4VreC1 deoR araΔ139 (ara-ura7)979 galU galK rpsL (Str+ endA1 supG) | Invitrogen |
| TG2               | F’ traD36 lacIq Δ (lacZ/M15 proA-B supE hsdS5 thi Δ (lac-pro) Δ (srl-rcA)305::Tn10 (Tet+)) | Bethesda Research Laboratories, USA |

Plasmids:

| Plasmids          | Relevant genotype or features of plasmid | Construction or Ref. |
|-------------------|----------------------------------------|----------------------|
| pAW70             | bla cat amyE::P₆-rsbR-rsbS-rsbT-rsbU   | 24                   |
| pMLK              | bla cat amyE::                         | 23                   |
| pET15b            | amp lacI P₆                                                      | Novagen |
| PETNRsbUΔ         | amp lacI P₆-rsbUΔ                                                                 | 26                   |
| PETNRsbUΔ         | amp lacI P₆-rsbUΔ                                                                 | This study |
| PTECrsbUΔ         | amp lacI P₆-rsbUΔ                                                                 | 26                   |
| pDG148            | bla kan pcoo lacI P₆-gene                                                      | 21                   |
| pGK01             | bla kan pcoo lacI P₆-gene::rsbU                                                 | This study |
| pGK02             | bla kan pcoo lacI P₆-gene::ΔrsbU1-38                                           | This study |
| pGK03             | bla kan pcoo lacI P₆-gene::ΔrsbU1-77                                           | This study |
| pGK04             | bla kan pcoo lacI P₆-gene::ΔrsbU1-93                                           | This study |
| pGK05             | bla kan pcoo lacI P₆-gene::ΔrsbU1-134                                           | This study |
| pMB21             | bla kan pcoo lacI P₆-gene::ΔrsbU1-19                                            | This study |

*The cat86 gene conferring chloramphenicol resistance is linked to the ctc::lacZ reporter gene fusion.

**The spe gene conferring resistance to spectinomycin was introduced by a Campbell-like recombination into the B. subtilis chromosome and is linked to the rsbUΔNΔdel allele.
IPTG-inducible expression of N- and C-terminal truncated RsbU variants was designed and their effect on activity was tested both in exponentially growing cells and in cells exposed to ethanol, a well known environmental stress stimulus. Specifically, the truncations were of the first 19, 38, 77, 93, and 134 amino acids. A wild-type copy and each of the truncated rsbU genes were cloned by PCR into the self-replicating plasmid pDG148 and thus were placed under the control of the IPTG-inducible promoter, P\text{lac} promoter. Plasmids were transformed into a derivative of B. subtilis wild-type strain BSA46, in which the chromosomal copy of rsbU had been inactivated by a deletion of an internal NdeI fragment in rsbU (BSA140) (14). The expression of all truncated versions of RsbU on IPTG induction was verified by Western blot analysis with monoclonal antibodies directed against RsbU (data not shown). Activity was monitored under the same conditions with a ctc-lacZ reporter gene fusion. Expression of the truncated RsbU variants was found to be strongly correlated to the growth of the wild-type strain and its derivatives (93). As expected, wild-type strain BSA46 displaying the ctc-lacZ fusion showed a significant increase in activity in the presence of IPTG (Fig. 2). The internal deletion in the ctc-lacZ reporter gene fusion, which is known to be strictly dependent (31) and thus the \( \sigma^B \)-dependent activity of those strains is directly correlated to the \( \sigma^B \)-dependent activity.

Surprisingly, although all the RsbU variants were expressed, none of the strains harboring the plasmid-encoded truncated rsbU alleles displayed any significant ctc-lacZ reporter gene activity during exponential growth in the presence of the inducer IPTG (data not shown). Only the strain carrying a plasmid-encoded full-length copy of rsbU displayed a modest \( \beta \)-galactosidase activity, and hence \( \sigma^B \) activity, in the presence of IPTG. Therefore, partial or complete deletion of the N-terminal domain of RsbU does not render RsbU constitutively active in vivo. Next, the ability of the truncated RsbU variants to mediate environmental stress-triggered activation of \( \sigma^B \) was tested. Cells grown in the presence or absence of the inducer IPTG were exposed to 4% ethanol, a well known strong inducer of the \( \sigma^B \) regulon (32, 33). As expected, wild-type strain BSA46 displayed strong transient induction of the ctc-lacZ fusion, which peaked 20 min after ethanol addition and was not altered by the inclusion of the inducer IPTG (Fig. 2). The internal deletion in the ctc-lacZ reporter gene fusion, which is known to be strictly dependent (31) and thus the \( \beta \)-galactosidase activity of those strains is directly correlated to the \( \beta \)-galactosidase activity in vivo.

RESULTS

Effect of Deletions of the N-terminal Domain of RsbU in Vivo—If the N-terminal domain of RsbU indeed acts as an inhibitory element preventing activation of RsbU in the absence of stress, partial or complete deletion of the N-terminal part of RsbU should render the phosphatase constitutively active. To test this hypothesis experimentally, a series of N-terminal truncated RsbU variants were designed and their effect on activity was tested both in exponentially growing cells and in cells exposed to ethanol, a well known environmental stress stimulus. Specifically, the truncations were of the first 19, 38, 77, 93, and 134 amino acids. A wild-type copy and each of the truncated rsbU genes were cloned by PCR into the self-replicating plasmid pDG148 and thus were placed under the control of the IPTG-inducible promoter, P\text{lac} promoter. Plasmids were transformed into a derivative of B. subtilis wild-type strain BSA46, in which the chromosomal copy of rsbU had been inactivated by a deletion of an internal NdeI fragment in rsbU (BSA140) (14). The expression of all truncated versions of RsbU on IPTG induction was verified by Western blot analysis with monoclonal antibodies directed against RsbU (data not shown). Activity was monitored under the same conditions with a ctc-lacZ reporter gene fusion. Expression of the truncated RsbU variants was found to be strongly correlated to the growth of the wild-type strain and its derivatives (93). As expected, wild-type strain BSA46 displaying the ctc-lacZ fusion showed a significant increase in activity in the presence of IPTG (Fig. 2). The internal deletion in the ctc-lacZ reporter gene fusion, which is known to be strictly dependent (31) and thus the \( \beta \)-galactosidase activity of those strains is directly correlated to the \( \beta \)-galactosidase activity in vivo.

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in after exposure to ethanol, but with a peak height that was roughly half that of BSA46. We do not consider that the difference in β-galactosidase activity between strains BSG14 and BSA46 is significant; rsbU is not under the control of P_{spe} in strain BSA46, unlike in strain BSG14, and the relative levels of RsbU in these strains, and hence the activity of ρ^4, may differ.

In an independent genetic approach, the promoter and polycistronic coding sequences for rsbR, rsbS, rsbT, and rsbU were cloned into pMLK (23). This plasmid, which permits integration at the amyE locus, was transformed into strain PB291 (24), in which rsbU had previously been deleted in a ctc-lacZ fusion background. This strain responds to ethanol shock in much the same way as BSA46 (data not shown), that is, it is independent of IPTG. A second strain was constructed that contained rsbR-rsbS-rsbT, but here a truncated copy of rsbU was cloned, corresponding just to the catalytic domain (residues 118–335), instead of the full-length gene. This particular gene product could not induce ρ^4 activity with or without the imposition of stress (data not shown).

RsbU provided in trans can thus convey environmental stress signals. In contrast, none of the strains carrying N-terminal truncated rsbU genes conferred constitutive activity on RsbU variants, nor could these strains activate ρ^4 in response to environmental stress. Thus any disruption to the N-terminal domain of RsbU, even a deletion of just the first 19 amino acids (Fig. 2B), is detrimental to its function and has profound effects on ρ^4 activation.

Comparison of the Phosphatase Activities of RsbU and its C-terminal Domain—The above results show that genetic deletions in the regions of rsbU that encodes its N-terminal domain lead to an inability of the cells to activate ρ^4 in vivo and so to respond to stress. These results suggest that the activation of the phosphatase function of RsbU has been affected by truncations in its N-terminal domain, and that the C-terminal domains created by deletion have no phosphatase activity. We addressed these points in vitro by measuring the phosphatase activity of the recombinant C-terminal domain of RsbU (C-RsbU, residues 118–335), and comparing it to the activity of RsbU alone. We also compared the phosphatase activity of C-RsbU and of RsbU each in the presence of a 2-fold molar excess of RsbT. The enzymatic assay revealed that while C-RsbU alone was active as a phosphatase toward RsbV-P, the rate of dephosphorylation of RsbV-P was only 4-fold greater than that of full-length RsbU in the absence of RsbT (Fig. 3). In contrast, the rate of dephosphorylation of RsbV-P by RsbU was ~40-fold higher when stimulated by the addition of a 2-fold molar excess of RsbT over RsbU (Fig. 3). C-RsbU displayed exactly the same, low phosphatase activity in the presence and absence of RsbT (Fig. 3).

The observation of diminished phosphatase activity of C-RsbU in vitro is consistent with our genetic data, which reveal that N-terminal truncated RsbU has insufficient phosphatase activity to trigger a ρ^4-directed response to stress. Therefore, it appears unlikely that the function of N-RsbU is to inhibit C-RsbU, with RsbT relieving this inhibition via an interaction predominantly, but not necessarily wholly, with the N-terminal domain of RsbU. We conclude that the N-terminal domain of RsbU does not exert an inhibitory influence on the C-terminal catalytic domain but rather is absolutely required for the activation of RsbU.

Interactions between RsbU and RsbT—In previous studies, the partner switching behavior of RsbW, RsbV, and ρ^4 was monitored by the use of non-denaturing polyacrylamide gel electrophoresis (16). We applied the same technique to study the interactions between different combinations of RsbT with RsbU, C-RsbU, and the N-terminal 112 residues of RsbU (N-RsbU-(1–112)), which was previously identified as a proteolytically stable fragment of RsbU (26). RsbT alone did not form a sharp, single band in native gels, and a significant quantity of material was found in the well of the gel (Fig. 4, lane 2). In contrast, RsbU and the isolated N- and C-terminal domains migrated well through the gel, with R_{f} = (relative mobility to the dye front) values of 0.41, 0.71, and 0.82 (Fig. 4, lanes 1, 3, and 4).

When RsbT was mixed with an excess of RsbU (Fig. 4, lane 5), an additional species was observed (R_{f} = 0.27) that migrated slower through the gel than RsbU, and which we presume is a complex of RsbT and RsbU. Similarly, an additional band R_{f} = 0.41, presumably corresponding to the complex of RsbT and N-RsbU-(1–112), was observed when these two proteins were mixed (Fig. 4, lane 6). However, when RsbT and C-RsbU were mixed (Fig. 4, lane 7), no additional species were observed, only those of the individual proteins. RsbT and RsbU are therefore capable of forming a complex stable enough to be observed in the conditions of gel electrophoresis. The data presented in Fig. 4 are consistent with the interaction between these two proteins being mediated predominantly, or solely, through the N-terminal domain of RsbU. The isolated domains of RsbU did not interact with each other under the electrophoresis conditions to reconstitute a "full-length" RsbU (Fig. 4, lane 8). It is thus unlikely that the two domains of RsbU form a stable intramolecular complex. Other, multidomain proteins whose structures undergo a conformational change as part of a regulatory mechanism cannot always be reconstituted from the purified individual domains. For instance, the two isolated...
domains of Spo0A do not interact under native gel electrophoresis conditions.

Stoichiometry and Stability of the RsbU-RsbT Complex—We have demonstrated that the presence of a 2-fold molar excess of RsbT over RsbU increased the latter’s phosphatase activity some 40-fold (Fig. 3). To determine the ratio of RsbU:RsbT that is required for maximum activation of RsbU, the rate of dephosphorylation of RsbV-P by RsbU was measured in the presence of increasing concentrations of RsbT. The phosphatase activity of RsbU increased linearly until the ratio between RsbT and RsbU reached 6:1, and reached its maximum at a ratio of 10:1 (Fig. 5). This maximum activity was ~200 times higher than that of RsbU in the absence of RsbT. Either RsbU binds 6 or more molecules of RsbT to gain maximum activation, or the ratio at which maximal activity is observed is a reflection of a low-affinity interaction between RsbT and RsbU.

Because it was possible by native gel electrophoresis to detect a complex between RsbU (or N-RsbU-(1–112)) and RsbT, the band corresponding to the RsbU-RsbT complex was excised from the gel, and the proteins within the band were electroeluted and then analyzed by SDS-PAGE. Bands corresponding to RsbU and RsbT were observed, and their relative intensities were measured after digitization of the gel. By comparison to known standards of RsbU and RsbT, we conclude that the stoichiometry of the RsbU-RsbT complex under these conditions is 1:1.

To characterize the interactions between RsbU and RsbT further, we used gel filtration chromatography to estimate the size of the purified individual proteins, isolated domains, and complexes. The same technique showed previously that RsbT is a monomer (18). The molecular mass of RsbU was found to be around 80 kDa, a value that corresponds to a dimer of RsbU composed of 38-kDa subunits (data not shown). This result is in close agreement with previous gel filtration experiments performed with a B. subtilis cellular extract from which RsbU eluted as a protein of ~90 kDa (34). The apparent molecular mass of the N-terminal 112 residues of RsbU was 34 kDa, suggesting that this molecule is dimeric, or perhaps trimeric. The size of the C-terminal construct of RsbU (residues 118–335, C-RsbU) predicted from its gene sequence is 25 kDa, which was consistent with the gel filtration elution profile that indicated that C-RsbU is monomeric. Hence it would appear that the dimerization determining motifs of RsbU are restricted to the N-terminal domain. Despite testing a variety of different conditions (pH, NaCl, ATP, and Mg2+ concentration), we could not isolate any complex by size exclusion chromatography, be it between RsbT and RsbU, or between RsbT and N-RsbU-(1–112). Given that the measured stoichiometry of the RsbU-RsbT complex is 1:1, yet RsbT must saturate RsbU for full activation, and that the RsbU-RsbT complex could not be isolated by gel filtration, these observations argue that there is only a weak affinity interaction between RsbU and RsbT.

Crystal Structure of N-RsbU-(1–112)—To understand further the function of the N-terminal domain of RsbU, the structure of N-RsbU-(1–112) was determined by x-ray crystallography, using the MAD technique from crystals of selenomethionyl-labeled N-RsbU-(1–112), and refined against diffraction data to 1.6 Å (Fig. 6A). Statistics of the diffraction data and final refined model are presented in Table II. The electron density map does not reveal the conformation of residues 85–112, and these amino acids are missing from the final structure; we assume them to be disordered. One molecule of N-RsbU-(1–112) comprises four anti-parallel α-helices, arranged into a rough “L” shape (Fig. 6B), forming a four-helical bundle. Such bundles are found commonly in biology in a variety of different circumstances; they are thermodynamically favored in protein folding, as seen in the globin superfamily, and also mediate interactions with themselves in dimer interfaces and with other molecules, such as DNA or proteins, for instance in Spo0B, homeodomains, and focal adhesion kinase.

To identify structural neighbors, the structure of N-RsbU was submitted to the DALI (35) server. More than 460 structures were identified as having structural similarity to N-RsbU, with Z-scores of 2.0 or greater. Not surprisingly, the sequence identities between N-RsbU and its structural neighbors were low, mostly less than 10%. No single identifiable biological theme linked the structures, and examples were found from the contexts where four helical bundles are prevalent (e.g. residues from hemoglobin (Z-score 3.1, Protein Data Bank code 1CGS),
Spo0B (2.5, Protein Data Bank code 1IXM), engrailed homodomain (3.2, Protein Data Bank code 2HDD), and FAK targeting domain (2.0, Protein Data Bank code 1K04).

Potentially relevant structural matches found by DALI include domain three of \(\sigma^b\) (\(\sigma_g\)) from Thermus aquaticus (2.1, Protein Data Bank code 1KU2), and representatives from the armadillo (\(\beta\)-importin, 4.5, Protein Data Bank code 1QGR; \(\alpha\)-catenin, 4.2, Protein Data Bank code 1DOV; \(\beta\)-catenin, 2.1, Protein Data Bank code 3BCT), HEAT (PP2A, 3.8, Protein Data Bank code 1B3U), and tetratricopeptide repeat (TPR) (PP5, 3.3, Protein Data Bank code 1AI7) families of helical repeat proteins; these are scaffolding molecules that mediate protein-protein interactions (36). \(\sigma_g\), which harbors residues that are important for the recognition of the \(-10\) region of the promoter, is also found in contact with SpoIIAB residues in the crystal structure of the \(\sigma^b\)-SpoIIAB complex (37). A recent study of the SpoIIAB homologue and RsbU activator, RabT, suggests that RabT residues Arg20-Gln40, Arg24-Asn24, and Asp50-Gln60 are involved in RsbT-RsbU interactions (38). These residues are equivalent to those in SpoIIAB that bind \(\sigma^b\). However, the SpoIIAB-contacting residues in \(\sigma^b\) have spatial equivalents in N-RsbU that are not solvent-accessible and thus can only bind RabT after significant conformational changes occur in N-RsbU. Therefore, the functional significance of the similarity between N-RsbU and \(\sigma_g\) remains to be elucidated.

On examination of the crystal packing, it was evident that N-RsbU-(1–112) had crystallized as a dimer around a crystallographic 2-fold axis. 28% of the available surface area, some 1600 \(\AA^2\), is buried in the dimer interface; 80% of the atoms in the interface are non-polar and no water molecules are observed in the interface at the resolution of 1.6 \(\AA\). This extensive dimer interface is composed of residues from all four helices of each protomer, but is dominated by helix 4, which contributes about half of the buried surface in the dimer interface, whereas helices 1 to 3 each contribute \(-1/6th\). Five hydrogen bonds between residues in protomer one and two are duplicated by the 2-fold symmetry of the interface, to make a total of 10 that stabilize the dimer. The residues involved, Gln31, Thr37, His51, Tyr55, Asp65, Ser69, Glu75, and Tyr80, are conserved across the RsbU family, except for Thr37 and the interacting pair of Tyr55 and Asp65 (Fig. 7). In this latter case, a change at one position in RsbU orthologues is compensated for by an alteration in the other position such that either hydrogen bonding or hydrophobic packing is conserved (e.g. pairings are found of Asp and Asn, or Gly and Phe, at positions 59 and 65 in the orthologues of RsbU). Variation to Leu, Phe, Ala, or Val is permitted at Thr37.
Analysis of the completed genomes of Gram

consider further the interaction between RsbU and RsbT. An

mutation in RsbU that affects activation by RsbT led us to

loss of dimerization of RsbU, affecting the binding surface on

dimer interface as well as to destabilize helix 3. The inability of

primates represented by blue bars. Residues colored green are totally con-

served across the RsbU family, those in red are conserved in those bacteria that encode rsbT, but not necessarily conserved in those bacteria that do not. The residue in black is Pro44, mutation of which to Arg suppresses a deletion in rsbX (39). Gaps in the alignment are repre-

sented by “-” residues that contribute to the dimer interface are high-

lighted by a * for hydrogen bond donors/acceptors and a ^ for hydro-

phobic packing. The sequences listed are B. subtilis (BACSU), Bacillus licheniformis (BACLI), Bacillus halodurans (BACHA), Gallionellus ichthyus (OBACI), L. monocytogenes (LIMON), Listeria innocua (LIINN), which all encode rsbT and S. aureus (STAURO) and S. epider-

midis (STEP1), which do not.

because residues in this position can interact with Tyr80 either by forming a hydrogen bond, or by hydrophobic packing. Other residues found at the dimer interface are well conserved across the RsbU orthologues sequenced to date.

In a previous study, the mutation P44R was identified in a genetic screen for suppressors of a deletion in rsbX (39). Strains deleted for rsbX exhibit constitutively high α helix activation, and rsbU44PR suppresses this activity. This mutation also abol-

ishes the activation of rsbX on the imposition of stress. In the structure of N-RsbU, Pro44 is situated at the dimer interface sandwiched between conserved hydrophobic residues including Met77 and Tyr84, and in conjunction with Glu45 it stabilizes the N-terminal end of helix 3. The data of Smirnova et al. (39) indicate that although rsbU44PR accumulates in the cell, it cannot be activated, because the ability of RsbU and RsbT to interact has been destroyed by this mutation. Mutation from proline to arginine at this position is highly likely to disrupt the dimer interface as well as to destabilize helix 3. The inability of

rsbU44PR to be activated by RsbT could be explained by the loss of dimerization of RsbU, affecting the binding surface on RsbU for the activator RsbT.

A Potential RsbT Binding Surface on N-RsbU.—The P44R mutation in RsbU that affects activation by RsbT led us to consider further the interaction between RsbU and RsbT. An

analysis of the completed genomes of Gram+ microorganisms that encode a α helix orthologue reveals that not all of the Rsb proteins are conserved. For instance RsbR, RsbS, RsbT, and RsbX have not been identified in the genomes of S. aureus and Staphylococcus epidermidis. Presumably, in those bacteria where no activator of RsbU has been discovered, RsbU is reg-

ulated in a different way to that in B. subtilis. Therefore, a comparison of the amino acid sequence of RsbU orthologues that originates from bacterial species that contain an RsbT activator, and those that do not, may reveal the RsbT-interact-

ing surface in RsbU. The sequence alignment of the N-terminal domains of RsbU orthologues (Fig. 7) provides insight into the structure and function of RsbU. Those residues that are com-

pletely conserved in the alignment are found at the dimer interface, participating in either hydrogen bond (e.g. His31 and Glu75), where ‘ ’ indicates that this residue is found in the other proteome in the dimer), or van der Waals (e.g. Tyr11, Leu72, and Leu73) contacts. Moreover, a larger amino acid than glycine at position 81, could not accommodate the approach of the long carboxylic acid side chain of Glu45, which also serves to N-cap helix 3. Other residues that are particularly poorly conserved are located mainly in loops between the secondary structural elements or on the solvent-exposed faces of the four helices.

The residues that are solvent-accessible and conserved in RsbT encoders (Bacilli and Listeriae), and not necessarily so in RsbT non-encoders (Staphylococci), are highlighted in the se-

quence alignment (Fig. 7). Glu45, Tyr80, and Arg84 are found in a discontinuous groove on one face of helix 2: the break at position 51 coincides with a 1-residue lysine insertion in the sequences of RsbU from the Staphylococci. In conjunction with the hydrophobic residues Ile74, Ile76, Met72 and Ala85 from helix 4, these residues form two prominent, parallel surface ridges of edge length −17 Å (Fig. 8A). These surface ridges are only formed as a consequence of the dimerization of N-RsbU and in effect they form a ~10-Å wide groove, the floor of which comprises residues Ser34, Ile86, Val79, Gly79, and Tyr80, which are well conserved across all RsbU orthologues. Overall, the prominent surface features of N-RsbU from B. subtilis are unlikely to be conserved in the Staphylococci, leading us to conclude that these surface patches may represent at least part of the binding surface for RsbT on RsbU. For instance, replace-

ment of Tyr80 by serine in the Staphylococci would undoubtedly alter the local structure and molecular surface. Calculation of the molecular surface after replacement of the tyrosine at this position by serine reveals that a cavity is formed (Fig. 8D).

A Comparison of RsbT Binding by N-RsbU-(1–84) and N-RsbU-(1–112)—In Fig. 4, we monitored the binding of RsbT by RsbU and N-RsbU-(1–112). However, the structure of N-RsbU-(1–112) did not reveal the conformation of residues 85–112, because this part of the protein was disordered in the crystal. Other residues that are conserved only in those bacte-

ria that code for rsbT include Glu86, Arg84, Ile87, and Ser99 (Fig. 7), which cannot be modeled in the present structure. These amino acids may also play a role in RsbT binding. To investi-

gate whether residues 85–112 of RsbU contribute significantly to RsbT binding, we constructed a truncated form of N-RsbU that corresponds precisely to the ordered portion of this crystal structure, i.e. residues 1–84. We monitored whether N-RsbU-

(1–84) could recruit RsbT in a binding assay, as analyzed by non-denaturing gel electrophoresis. N-RsbU-(1–84) runs a little quicker in electrophoresis than N-RsbU-(1–112) in the absence of RsbT, but in its presence an additional electrophoretic species can be observed for both constructs of N-RsbU, which we conclude corresponds to N-RsbU-RsbT complexes (Fig. 9a). Note that the band corresponding to the N-RsbU-(1–84)-RsbT complex in lane 1 is slightly more diffuse than the band in lane 2 of the N-RsbU-(1–112)-RsbT complex. The relative diffuse-

ness may reflect the fact that the interaction of RsbT with N-RsbU-(1–84) is slightly weaker than that between RsbT and N-RsbU-(1–112).

To assess whether there is a significant difference in the bind-

ing affinity of the two N-RsbU constructs for RsbT, we designed a competition assay in which a molar equivalent of N-RsbU domains in comparison to RsbU was added to RsbV-P dephosphorylation reactions. The initial rate of RsbV-P dephosphorylation was reduced by 45% for N-RsbU-(1–84) and 40% for N-RsbU-(1–112) (Fig. 9b). The results of these experiments indicate that both N-RsbU-(1–84) and N-RsbU-(1–112) are compe-

tent in binding RsbT, and that the major RsbT-binding determinants are found in those amino acids that could be located in the
crystallographic electron density, residues 1–84. Although we cannot rule out the possibility that residues 85–112 are involved in the binding of RsbT during the activation of RsbU, we can conclude that any interaction must be relatively weak.

**DISCUSSION**

PP2C-type phosphatases act in the signaling pathways that regulate the response to stress in eukaryotes (40, 41) as well as in *B. subtilis* and its close relatives (7). The *B. subtilis* PP2C-type phosphatase, RsbU, activates the general stress σ factor, σH, following the imposition of environmental stress (see Fig. 1). Induction of the large σH-dependent regulon provides the cell with a multiple and pre-emptive stress resistance (42), but also constitutes a considerable burden. Thus, tight control of the activity of σH, and therefore RsbU, is crucial. The activities of eukaryotic phosphatases are known to be controlled by a variety of mechanisms, including post-translational modification and the binding of accessory proteins, but little is known of the regulation of PP2C phosphatases (43). For instance, the eukaryotic cell cycle phosphatases Cdc25A (44) and Cdc25C (45) are activated by phosphorylation. In contrast, the kinase activity of RsbT is not required for activation of RsbU (19).

A simple activation mechanism for RsbU, where the N-terminal domain suppresses the function of the C-terminal domain until a conformational change occurs in RsbU on the binding of the activator, RsbT, would be an elegant solution to the problem of control. Such a mechanism has already been observed for human and rabbit protein phosphatase 5 (PP5), which is stimulated by the interaction of arachidonic acid with the TPR domain of PP5. This interaction drives a conformational change in PP5 that overcomes the TPR-mediated inhibition of the phosphatase domain. In this instance, the enzymatic activity of arachidonic acid-stimulated PP5 approaches

![Figure 8](image-url)  
*Fig. 8. The RsbT-binding surface?*  
A, the molecular surface of the N-RsbU dimer is colored mostly blue: those parts of the surface colored lime correspond to residues that are completely conserved in the sequence alignment in Fig. 7; note that few of them map to the surface, and the ones that do (Leu27 and Glu75; Gly81 and Glu45) are part of the dimer interface, situated between helices 3 and 4. The red patches of the molecular surface relate to those amino acids that are conserved in bacteria that encode RsbT in their genomes, but are not necessarily conserved in bacteria that do not harbor an *rsbT* gene. B, the inset reveals a section of the surface in the vicinity of Tyr18, where the tyrosyl side chain has been replaced with a seryl, with the result that a cavity is formed. In both panels, the same orientation as panel A in Fig. 6 is used, and this figure was prepared with PyMOL (www.pymol.org).

![Figure 9](image-url)  
*Fig. 9. An analysis of the protein-protein interactions between RsbT and N-RsbU.*  
A, a native gel binding assay, as described for Fig. 4, where the ability of RsbT to bind to N-RsbU-(1–84) and N-RsbU-(1–112) was monitored. Lane 1, N-RsbU-(1–84) and RsbT; lane 2, N-RsbU-(1–112) and RsbT. The position of the free individual proteins are marked with an arrow, protein-protein complexes are marked with an asterisk. Each lane contains ~10 μg of total protein. B, a competition assay in which the initial rate of RsbV-P dephosphorylation by 0.5 μM RsbU stimulated by 0.5 μM RsbT was measured in the presence of 0.5 μM of the two N-RsbU constructs. Along the x-axis in lane 1, RsbU alone; lane 2, RsbU stimulated by RsbT; lane 3, RsbU stimulated by RsbT in the presence of N-RsbU-(1–84); lane 4, RsbU stimulated by RsbT in the presence of N-RsbU-(1–112). The y-axis corresponds to the percentage of RsbV-P dephosphorylation activity normalized to RsbU stimulated by RsbT. The results shown are the average of three independent experiments and the error bars represent the S.D. ± mean.
that of the isolated phosphatase domain (46). However, in vitro the isolated catalytic domain of RsbU has a very low phosphatase activity in comparison to RsbT-activated full-length RsbU (Fig. 3). Moreover, N-terminal truncated RsbU proteins cannot induce \( \sigma^B \) activity in vivo, and furthermore, the basal level of \( \sigma^B \) activity in these strains resembles that of wild type (Fig. 2). The N-terminal domain of RsbU is therefore absolutely required for RsbU activation, by recruiting RsbT (Fig. 3).

The results presented here support and extend the earlier conclusions by Kang et al. (19) that RsbT activates RsbU by forming a protein-protein complex. In this study, we demonstrate that the association is mediated predominantly, but not necessarily exclusively, by the first 84 amino acids of the N-terminal domain of RsbU (Figs. 4 and 9). The crystal structure of the N-terminal domain of RsbU reveals that only a dimeric form of RsbU can form what we believe to be an RsbT-binding surface. Deletion of the N-terminal part of rebU (this study), or mutation of Pro44 in the dimer interface (39), has deleterious effects on the stress response of B. subtilis, presumably because the dimer form of RsbU is destroyed. The residues that would appear to mediate these protein-protein interactions are only conserved in RsbU orthologues from bacteria that include rebT in their genomes (Fig. 7).

What molecular mechanism is used in the activation of the phosphatase domain of RsbU during the recruitment of RsbT by N-RsbU? RsbU is dependent on manganese, and one method of RsbU activation might involve the modulation of the \( K_m \) for Mn\(^{2+} \) in the presence of RsbT, leading to a more efficient dephosphorylation of RsbV-P. This model is not without precedent in the PP2C phosphatase family; the catalytic subunit of bovine mitochondrial pyruvate dehydrogenase phosphatase displays a \( K_m \) for magnesium that increases in the presence of its regulatory subunit (47). However, the \( K_m \) value for Mn\(^{2+} \) of RsbU, of RsbU in the presence of RsbT, and of the C-terminal domain of RsbU alone were found to be 0.96 M in all three cases (data not shown) and thus similar to that observed for other PP2C phosphatases (48, 49). It would therefore seem unlikely that RsbU is regulated by changes in the \( K_m \) for its requisite co-factor.

An alternative mechanism might entail the modulation of the oligomeric state, a common means of control. From solution measurements, we have concluded that RsbU, in the absence of RsbT, is a dimer, and the structure of N-RsbU reveals a significant dimer interface (Fig. 6). The thermodynamic barrier of dimer dissociation in this instance is likely to be considerable. Unless the reaction is driven by the free energy of hydrolysis of ATP, for instance, the apparent robustness of the N-RsbU dimer interface should exclude an activation mechanism where the presence of RsbT dissociates the N-RsbU dimer, leading to enzymatic stimulation.

Alternatively, the formation of the RebT-RsbU binary complex might be a prerequisite for phosphatase activation. As in N-RsbU, the non-catalytic domains from phosphatases 2A (50), 2C (51), and 5 (52) are also all \( \alpha \)-helical (Fig. 10). Despite the fact that there is little meaningful sequence homology between these domains and RsbU, these non-catalytic domains were all found with DALI, with Z-scores of 3.8 (PP2A), 3.2 (PP2C), and 2.2 (PP5). For instance, helices 1, 3, and 4 of N-RsbU were aligned against all three helices in the regulatory domain of PP2C, which has been proposed to play a role in the determination of substrate specificity (51). Similarly, N-RsbU also matched to the TPR motifs from PP5, which are used to mediate macromolecular interactions and the assembly of protein-protein complexes. Furthermore, there is structural similarity between N-RsbU and the HEAT repeat units of PP2A, which coordinate the assembly of the phosphatase and regulatory subunits into a functional heterotrimer (50). It appears that the N-terminal domain of RsbU may have evolved to perform a similar scaffolding function, recruiting RebT, and perhaps it is this binary complex that actually recognizes the RebV-P substrate.

In the absence of a stable RebT-RsbU complex, determination of the molecular basis of phosphatase activation has proven elusive. The present study reveals that for maximal activation of RsbU, at least a 6-fold molar excess of RsbT must be present (Fig. 5). We have previously demonstrated that it is likely that there are at least six molecules of RsbT bound per stressosome prior to the induction of stress signals (18). It is perhaps not coincidental that the same number of molecules of RsbT that is sequestered by the stressosome is required to activate fully RsbU. The expression of \(~130 \sigma^B\)-dependent genes in response to stress is an energetically demanding process, and therefore has to be kept under strict control. The partnership between the stressosome and RsbU may actually sense the amplitude of the stress signal, and act as a tuning system to regulate the level of \( \sigma^B \) activity in accordance to the level of stress. This partnership could also act as part of a damping mechanism, if a threshold level of RsbU activity is necessary to induce \( \sigma^B \).
activity, ensuring that a stress-response is triggered only under certain conditions. RsbX, the phosphatase for Rsb-P, is required to restrict the activity of αβ to pre-stress levels after the imposition of stress (53). In contrast to the stable interactions observed between RsbW and αβ or RsbV, relatively weak interactions between RsbT and RsbU may have evolved to permit the rapid and transient activation of the αβ-dependent general stress regulon that reaches its peak within 20 min in laboratory conditions.

This study reveals that the N-terminal domain of RsbU shares structural similarity to non-catalytic domains from protein phosphatase families 2A, 2C, and 5. We propose that the structural similarity extends to a functional one. The phosphatase regulatory domains are found across the plant and animal kingdoms, and it is thus likely that these phosphatases share a common mechanism of control with RsbU, the binding of an additional regulatory subunit. The structural similarity between N-RsbU and αβ may have functional as well as evolutionary significance. RsbT is related to a group of anti-σ factors that inhibit σ factor activity by binding to αβ. The partner switching of RsbT and RsbU appears to have developed in parallel to that of α and anti-σ factors, to use similar structural motifs in complex formation. Unlike the robust ασ anti-σ factor complexes, however, the molecular surfaces involved in the formation of the RsbU-RsbT complex have evolved mutually to weaken their interaction as part of the transient ασ-activation mechanism.

Nonetheless, several questions remain unanswered. Does the ephemeral association of RsbT with RsbU drive a relatively long-lived conformational change in RsbU that is necessary for activation, or is the RsbT-RsbU binary complex active as a phosphatase? If so, is there a measurable interaction of the binary complex with the RsbP substrate? Experiments are underway to answer these questions, and to confirm if the residues we identified by genomic sequence analysis are utilized in the recruitment of RsbT by RsbU.

Acknowledgments—We thank Chet Price for the gift of strain PB291 and plasmid pAW70, and William G. Haldenwang for providing the monoclonal antibodies directed against RsbU. We are grateful for access to the ESRF, and beamline support from Martin Walsh (BM14) and Jan Pane. We were alerted by (and are grateful to) Dr. Alexey Murzin to the structural and functional similarity between N-RsbU and KaiC. We also thank Harry Gilbert and assistants at the ESRF, and beamline support from Martin Walsh (BM14) and Deloumeau, O., Lewis, R. J., and Yudkin, M. D. (2002) J. Bacteriol. 184, 5583–5589.

Note Added in Proof—We were alerted by (and are grateful to) Dr. Alexey Murzin to the structural and functional similarity between N-RsbU and the C-terminal domain of the clock protein KaiA. Both domains form similar dimers, and the KaiC-binding site in C-KaiA (see Vakonakis, I., and Li Wang, A. C. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 10925–10930) is in the equivalent location to the proposed RsbT binding site on the N-RsbU dimer.
Functional and Structural Characterization of RsbU, a Stress Signaling Protein Phosphatase 2C
Olivier Delumeau, Sujit Dutta, Matthias Brigulla, Grit Kuhnke, Steven W. Hardwick, Uwe Völker, Michael D. Yudkin and Richard J. Lewis

J. Biol. Chem. 2004, 279:40927-40937.
doi: 10.1074/jbc.M405464200 originally published online July 19, 2004

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