Interactions between the PTS Regulation domains of the BglG Transcriptional Antiterminator from Escherichia coli

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The E. coli BglG protein inhibits transcription termination within the bgl operon in the presence of β-glucosides. BglG represents a family of transcriptional antiterminators that bind to RNA sequences, which partially overlap rho-independent terminators, and prevent termination by stabilizing an alternative structure of the transcript. The activity of BglG is determined by its dimeric state, which is modulated by reversible phosphorylation catalyzed by BglF, a PTS permease. Only the non-phosphorylated BglG dimer binds to RNA and allows read-through of transcription. BglG is composed of three domains: an RNA-binding domain followed by two domains, PRD1 and PRD2 (PTS regulation domains), which are similar in their sequence and folding. Based on the three-dimensional structure of dimeric lacI, an E. coli homologue from Bacillus subtilis, the interactions within the dimer are PRD1-PRD1 and PRD2-PRD2. We have shown before that PRD2 mediates homodimerization very efficiently. Using genetic systems and in vitro techniques that assay and characterize protein-protein interactions, we show here that the PRD1 dimerizes very slowly, but once it does, the homodimers are stable. These results support our model that formation of BglG dimers initiates with PRD2 dimerization followed by zipping up of two BglG monomers to create the active RNA-binding domain. Moreover, our results demonstrate that PRD1 and PRD2 heterodimerize efficiently in vitro and in vivo. The affinity among the PRDs is in the following order: PRD2-PRD2 > PRD1-PRD2 > PRD1-PRD1. The interaction between PRD1 and PRD2 offers an explanation for the requirement of conserved residues in PRD1 for the phosphorylation of PRD2 by BglF.

Transcription of the bgl operon, whose products are required for β-glucoside utilization, begins constitutively, but in the absence of β-glucosides, transcription terminates at one of two rho-independent terminators that flank the first gene in this operon, bglIG. However, in the presence of β-glucosides, transcription proceeds through the putative terminators, the operon is expressed, and the β-glucosides are transported into the cell, phosphorylated, and metabolized (1). This pattern of expression is regulated by two of the bgl operon products, BglG and BglF. BglG is an RNA-binding protein, which binds to a sequence that partially overlaps each of the terminators and stabilizes an alternative RNA structure that enables the RNA polymerase to proceed (2). BglG also binds to the β'-subunit of RNA polymerase, but it is not known whether this binding is required for antitermination or for subsequent transcriptional steps (3). BglF, an enzyme II of PTS,1 is a membrane-bound sugar sensor that regulates the activity of BglG by reversibly phosphorylating it according to β-glucoside availability (4, 5). We have also demonstrated that the phosphorylation state of BglG determines its oligomeric state (6). Hence, in the absence of β-glucoside, BglG exists in the cell as a phosphorylated inactive monomer, whereas in the presence of the sugar in the growth medium, BglG exists as an active non-phosphorylated dimer. We have recently shown that in the absence of the stimulating sugar, BglF recruits BglG to the membrane, releasing it to the cytoplasm upon the addition of β-glucoside (7).

BglG represents a growing family of transcriptional antiterminators that regulate expression of genes and operons whose products are required for utilization of PTS carbohydrates in a variety of organisms (see Ref. 8 and references therein). The activity, regulation, and structure of some members of this family were investigated. The BglG-like proteins are usually composed of three domains, an RNA-binding domain followed by two reiterated domains designated PRD1 and PRD2 (PTS regulation domains). Four histidines, two in each PRD, are conserved, although BglG lacks the fourth histidine. Multiple sequence alignment of representatives of this family and a schematic presentation of BglG are shown in Fig. 1. The conserved histidines are essential for regulation of the antiterminator proteins by the PTS. On one hand, HPr positively regulates many of these regulators by phosphorylating one or more of the conserved histidines. The HPr-catalyzed phosphorylation was proposed to be part of a mechanism of carbon catabolite repression (CCR) that operates mainly in Gram-positive bacteria (9). On the other hand, enzymes II (EIIs) of PTS, such as BglF, negatively regulate the activity of the antiterminators by phosphorylating one of the conserved histidines. The site on BglG that is phosphorylated by BglF was mapped to His-208 in PRD2 (10). Nevertheless, two conserved residues in PRD1 (highlighted in Fig. 1A), Asp-100, which is adjacent to the first conserved histidine, and His-160, the second conserved histidine, are also required for negative regulation of BglG by BglF (11) and their replacement leads to constitutive expression of the bgl operon. Why are residues in PRD1 required for phosphorylation of PRD2? One possibility could be that in the three-dimensional structure, these residues, and hence PRD1 and PRD2, come

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together to form the site of recognition or phosphorylation by BglF.

The structure of the N-terminal RNA-binding domain of SacY and LicT was determined (12–14). These domains form dimers at relatively high salt concentrations. For SacY, it was reported that lowering the ionic strength resulted in dissociation of the dimers and their refolding was hard to accomplish (12). The RNA-binding domain of BglG cannot dimerize in vivo (15). Therefore, the RNA-binding domain of the BglG-like antiterminators was not predicted to mediate their dimerization under physiological salt concentrations. Instead, the C terminus, which contains the PRDs, was predicted to mediate dimerization and bring the two RNA-binding domains together. Using the one-hybrid ACI-based system, which relies on the DNA-binding domain of the CI repressor from phage λ as a reporter for dimerization (16), we have shown before that PRD2 of BglG dimerizes very efficiently (15). Using this genetic system, we failed to detect significant dimerization of truncated BglG molecules that lacked PRD2. Therefore, we suggested that formation of the active BglG dimers is mediated by PRD2 followed by zipping-up of the monomers to bring the two N-terminal RNA-binding domains together (15). The structure of the region that contains the two PRD domains of a LicT mutant, in which two of the conserved histidines were replaced by aspartates, was solved by x-ray crystallography (17). The structure is a homodimer, each monomer containing two analogous α-helical domains. The two PRDs adopt a similar structure, and the interactions within the dimer are PRD1-PRD1 and PRD2-PRD2. The phosphorylation sites are totally buried at the dimer interface and are hence inaccessible to the (de)phosphorylating enzymes, suggesting a major conformational change upon reversible phosphorylation or a significant difference between the mutant structure and the actual structure of the wild-type protein. Thus far, a structure of a monomer of any of these proteins has not been solved.

In this study, we used bacterial one- and two-hybrid systems and two in vitro techniques, Far-Western and surface plasmon resonance (SPR), which assay and characterize protein-protein interactions, to show that the PRD1 forms homodimers very slowly as opposed to PRD2. The phosphorylation sites are totally buried at the dimer interface and are hence inaccessible to the (de)phosphorylating enzymes, suggesting a major conformational change upon reversible phosphorylation or a significant difference between the mutant structure and the actual structure of the wild-type protein. Thus far, a structure of a monomer of any of these proteins has not been solved.

In this study, we used bacterial one- and two-hybrid systems and two in vitro techniques, Far-Western and surface plasmon resonance (SPR), which assay and characterize protein-protein interactions, to show that the PRD1 forms homodimers very slowly as opposed to PRD2. However, once PRD1 homodimers are formed, they are stable and their rate of dissociation is slow. These results support our model that dimerization of BglG initiates by PRD2 dimerization followed by zipping up of two BglG monomers to create an active dimeric RNA-binding domain. Moreover, we show here that the affinity between PRD1 and PRD2 is high and they heterodimerize efficiently in
interactions between the PRD Domains of BglG

Plasmids used in this study and the proteins they encode

| Plasmid | Plasmid-encoded protein | Source or reference |
|---------|-------------------------|---------------------|
| pJH157 | αCI | 18 |
| pJH91 | αCDDBB-LZ(GCN4)-β-galactosidase | 35 |
| pOAC100 | αCDDB | 6 |
| pOAC101 | αCDDBB-BglG | 6 |
| pLFPRD1 | αCDDBB-BglG PRD1 (aa 65–172) | 15 |
| pAB104 | αCDDBB-BglG PRD2 (aa 175–378) | 15 |
| pMS604 | LexAαDBD(WT)-Fos Leu zipper | 18 |
| pDP804 | LexAαDBD(mutant)-Jun Leu zipper | 18 |
| pLL1 | LexAαDBD(mutant) | 7 |
| pLL3 | LexAαDBD(mutant)-BglG | 7 |
| pLL3-G | LexAαDBD(mutant)-BglG | 7 |
| pLF1-PRD1 | LexAαDBD(mutant)-BglG PRD1 (aa 65–172) | 6 |
| pLF3-PRD1 | LexAαDBD(mutant)-BglG PRD1 (aa 65–172) | 6 |
| pLF1-PRD2 | LexAαDBD(mutant)-BglG PRD2 (aa 175–278) | 7 |
| pLF3-PRD2 | LexAαDBD(mutant)-BglG PRD2 (aa 175–278) | 7 |
| pANSN-PRD1 | HisαBglG PRD1 (aa 65–172) | 6 |
| pANSN-PRD2 | HisαBglG PRD2 (aa 175–278) | 6 |
| pANS5M-PRD1 | MBP- BglG PRD1 (aa 65–172) | 6 |
| pANS5M-PRD2 | MBP- BglG PRD2 (aa 175–278) | 6 |

Plasmids for which no reference has been cited were constructed as part of this work.

αCDDBB is a fragment of the CI repressor (amino acids 1–131), which contains a domain that binds to the PhoP and PhoQ operators on the λ genome.

LexAαDBD(WT) is a fragment of wild-type LexA repressor (amino acids 1–87), which contains a domain that binds to wild-type LexA operator.

LexAαDBD(mutant) is a fragment of LexA 488 repressor (amino acids 1–87), which contains a domain that binds to an altered LexA operator (op 45).

vivo and in vitro. The hierarchy of the interactions between the PRDs with regard to the stability of the interactions is PRD2 > PRD1. The interaction between PRD1 and PRD2 offers a rationalization for the requirement of two conserved residues in PRD1 for the phosphorylation of BglG-PRD2 by BglIF. The PRD1-PRD2 interaction might also explain why PRD2 mediates dimerization better than the entire BglG protein.

EXPERIMENTAL PROCEDURES

Strains—The following E. coli K-12 strains were used: AG1688 [MC1061 (F’ lacI’ lacZΔM15)] (16), a λ-sensitive bglI strain, was used to test for sensitivity to the cI- phage λKH54. JH372 (AG1688 λ202)) (16), which carries the ΔcI-leu23Z chromosomal fusion, was used to measure in vivo binding to λO41 (see below). SU202, which harbors a chromosomal copy of the lacZ gene under the control of a hybrid LexA operator (op408(αp+)) (18) was used to study heterodimerization. BL21(DE3) [hsdS gal (λcI857 ind 11 galT17 lacUV57 tet1 gene1)], obtained from Nogenel, and MC1061 [hsdR, mcrB, araD, 1534 araBAD lacI Q1675 lacX74 galU galK lacZ16thi] were expressed for expression of His- and maltose-binding protein (MBP)-tagged proteins, respectively.

Chemicals—N-Hydroxyquinicinamide (NHS), N-ethyl-N-(3-diethylaminopropyl)carbodiimide (EDC), ethanolamine hydrochloride, and HBS buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, and 0.06% P-20) were obtained from BIAcore AB (Uppsala, Sweden). Carboxymethyl dextran was obtained from Fluka Chemie. IPTG, indasaline, DNa, RNase, and maltose were obtained from Sigma. AEBSF was obtained from Calbiochem. Ni-nitroliotricarboxylic acid (NITcA)-NTA resin was obtained from Qiagen. Amylose resin and MBP (maltose-binding protein) were obtained from New England Biolabs. Anti-His monoclonal antibodies were obtained from Amersham Biosciences. Peroxidase-conjugated AffiniPure goat anti-mouse and goat anti-rabbit IgG (H+L) were obtained from Jackson ImmunoResearch Laboratories Inc.

Plasmids—All of plasmids used in this study and the proteins they encode are listed in Table I. Plasmids used for αCI-based system are derivatives of pJH391, which express the DNA-binding domain of CI fused to the leucine zipper domain of GCN4, which contains an insertion of truncated β-glucosidase (16). Plasmids used for the LexA-based two-hybrid system are derivatives of pLL1 and pLL3 that were constructed from pMS604 and pDP804, which express a LexAα-WT-Fos zipper fusion and a LexAα-408-Jun zipper fusion (18), respectively, by deleting the fragments that encodes the Fos and Jun zippers and introducing convenient restriction sites. Plasmids used for Far-Western and SPR were constructed by cloning the sequences encoding amino acids 65–172 of BglG (PRD1) or amino acids 175–278 of BglG (PRD2) in pET15b (Novagen) or in pET6b1, a derivative of MBP-αp2 (19). The hierarchy of the interactions between the PRDs with regard to the stability of the interactions is PRD2 > PRD1. Because in this phage OX1 contains an OX2 mutation, the intact λ repressor does not show cooperative binding. Its activity can be readily compared with the activity of the chimeric proteins derived from it. For a quantitative assay of heterodimerization, SU202 strain, which harbors a chromosomal copy of the lacZ gene under the control of a hybrid LexA operator (op408(αp+)), was co-transformed with the derivatives of pDP804 and pMS604 (18).

Phages—For αCI-based system, wild-type αCI was used. For Far-Western analysis, ESF5 was used instead of PMSF.

Surface Plasmon Resonance—SPR measurements were performed with a BIAcore3000 system (BIAcore AB). All of the procedures were performed at 25 °C. His-tagged BglG domains, PRD1 and PRD2 (5 μM/ml), in 10 mM sodium acetate, pH 5.5, were immobilized on the dextran surface of CM5 sensorchips by the standard amino coupling method (23) with the exception that the surface was derivatized with 11 μl with the Ni-NTA resin. AEBSF was used during the purification process instead of PMSF.

Far-Western Analysis—Proteins were separated on 10% SDS-polyacrylamide gels. Gels were subjected to Far-Western analysis as described previously (5) or stained with Coomassie Blue.

RESULTS

Studies of the Interactions between the PRD Domains of BglG by One- and Two-hybrid Systems—We have previously established BglG dimerization in vivo using the region coding for the DNA-binding domain of the CI repressor of bacteriophage λ (ADBD) as a reporter for dimerization (6). We have also shown that PRD2 alone can mediate ADBD dimerization efficiently (15). When a truncated BglG protein that contains the RNA-binding domain and the entire PRD1 domain but lacks most of PRD2 was fused to ADBD, it did not mediate ADBD dimerization (15). However, this truncated BglG protein had a partial
antitermination activity in vivo, approximately 35% of the entire BglG protein activity. Because dimerization is a prerequisite for antitermination, this result implied that the truncated BglG protein can form dimers, albeit not very efficiently. To address the question to what extent PRD1 is involved in mediating BglG dimerization, we fused the PRD1 domain to λDBD and compared its ability to mediate λDBD dimerization with the ability of the PRD2 domain and the entire BglG protein, each fused to λDBD. The ability of the plasmid-encoded chimeric proteins to dimerize was indicated by their ability to protect their bacterial hosts against λ infection and to repress expression of a $\lambda P_{\text{c}-\text{lacZ}}$ chromosomal fusion. The λDBD alone (amino acids 1–131 of λCI repressor), which cannot dimerize, served as a negative control in this assay. All of the proteins were expressed at low concentrations (without promoter induction) from equivalent plasmid constructs. The results presented in Table II clearly demonstrate that, whereas bacterial cells that expressed λDBD-PRD2 were immune to infection by bacteriophage λ and exhibited 80% repression of $\lambda P_{\text{c}-\text{lacZ}}$ expression as compared with 71% repression conferred by the entire BglG fused to λDBD, bacterial cells that expressed λDBD-PRD1 poorly repressed $\lambda P_{\text{c}-\text{lacZ}}$ expression (23% repression), although this extent of dimerization was sufficient to confer immunity against λ infection. These results show that, whereas the PRD2 domain mediates dimerization very efficiently, the PRD1 domain dimerizes weakly. The similarity between the two PRD domains together with the results, which show better dimerization of PRD2 than that of the entire BglG protein and poor dimerization of PRD1, raises the possibility that PRD1 inhibits PRD2 dimerization in the entire protein by interacting with it.

The λCI-based system enabled us to study homodimerization of the two PRD domains. To test whether PRD1 and PRD2 can form heterodimers in vivo, we used the LexA-based bacterial two-hybrid system (18). In this system, the proteins of interest are fused either to the DNA-binding domain of wild-type LexA repressor (LexA(DBD)) or to an altered specificity LexA(DBBD) and introduced into a strain that harbors a chromosomally copied lacZ under the control of a LexA-hybrid operator. Transcriptional repression is achieved upon co-expression of both hybrid protein, provided that they bind to each other. We first tested the ability of each PRD domain to dimerize with itself and then the ability of PRD1 and PRD2 to dimerize with each other. The results are presented in Table III. The leucine zipper domains of Fos and Jun fused to the wild type and mutant LexA(DBD), respectively, and fusions between BglG and the two LexA(DBBDs) served as positive controls (99 and 88% repression). The two LexA(DBBDs) served as a negative control (0% repression). When PRD2 was fused to both wild type and mutant LexA(DBD), 78% repression was obtained, substantiating the ability of PRD2 to form homodimers in vivo. When PRD1 was fused to both wild type and mutant LexA(DBBD), 56% repression was obtained, demonstrating again that the PRD1 homodimers are less stable than the PRD2 homodimers. The different values obtained for PRD1 homodimerization by the λCI-based and the LexA-based genetic systems can be explained by the difference in the levels of the fusion proteins. Because at high concentrations, the λDBD is able to dimerize (24), the concentrations of the chimeric protein are kept low by conducting the experiment in a lacI strain in the absence of an IPTG inducer. In contrast, the use of IPTG is recommended for the LexA-based system (18) and therefore low affinity interactions are manifested more profoundly in this case.

Co-expression of PRD1 fused to the wild-type LexA(DBD) and PRD2 fused to mutant LexA(DBBD) resulted in a stable heterodimer that functioned as a repressor, as indicated by the 79% transcriptional repression of lacZ expression from the LexA-hybrid operator (Table III). The stability of the PRD1-PRD2 heterodimer is comparable with that of the PRD2-PRD2 homodimer (79 and 78% repression, respectively).

Taken together, the results obtained with the two genetic systems demonstrate that in addition to the ability of PRD2 to form stable homodimers in vivo it can also form stable heterodimers with PRD1. The PRD1 domain, on the other hand, can dimerize in vivo albeit poorly.

In Vitro Studies of the Interactions between the PRD Domains of BglG—To examine the interactions of the PRD domains with themselves and with each other directly, we tested the interactions among purified PRD domains in vitro by the Far-Western technique. Similar amounts of purified PRD1 and PRD2 fused to MBP (MBP-PRD1 and MBP-PRD2, respectively) were subjected to SDS-PAGE (Fig. 2A, Coomassie Blue stain) and blotted onto two nitrocellulose filters. One membrane was incubated with His-tagged PRD1, and the other was incubated with His-tagged PRD2. Both membranes were then incubated with antibodies against the His tag. The results are presented in Fig. 2B. The weakest signal was detected for the interaction of PRD1 with itself (MBP-PRD1 on gel probed by His-PRD1, Fig. 2B, lane 1). The strongest interaction was obtained for PRD2 with itself (MBP-PRD2 on gel probed by His-PRD2, Fig. 2B, lane 5). A strong interaction between PRD1 and PRD2 was detected both when MBP-PRD1 was probed with His-PRD2 (Fig. 2B, lane 3) and when MBP-PRD2 was probed with His-PRD1 (Fig. 2B, lane 4). When MBP alone was probed with either His-PRD1 or His-PRD2, no binding was observed (Fig. 2B, lanes 3 and 6, respectively), indicating that the MBP moi-
Interactions between the PRD Domains of BglG

To study the kinetics of the interaction of the PRD domains with themselves (PRD1-PRD1 and PRD2-PRD2) and with each other (PRD1-PRD2), we used SPR, which enables the measurement of $k_a$ and $k_d$. Both PRD domains were immobilized on a sensorchip, each on a different channel, leaving one-flow cell blank, and various concentrations of PRD1 or PRD2 from 0.625 to 10 $\mu$m were passed over the chip. The response from the reference surface was subtracted from the response in each channel with the PRDs to give a signal (resonance units, RU) that is directly proportional to the amount of bound PRD. The association and dissociation constants were assessed by analysis of the kinetic data, and an equilibrium constant ($K_D$) was calculated from the apparent kinetic constants by fit to a first-order kinetic model. A single sensorgram curve for each channel obtained at 5 $\mu$m PRD1 or PRD2 is presented in Fig. 3.

A visual comparison of the four sensorgrams shows that there is binding in all cases. However, it is quite evident that the affinity of PRD2 to itself is higher (Fig. 3A) than the affinity of PRD1 to itself (Fig. 3B). The rate of PRD2 association with itself is high, and the PRD2-PRD2 complex is stable as indicated by the $K_D$ value for the PRD2-PRD2 complex (2.2 $\times$ 10$^{-8}$). Notably, after the completion of the injection, the decay of the binding signal for the PRD2-PRD2 pair was extremely slow, indicating that in addition to the high affinity, the PRD2-PRD2 complex is highly stable (the PRD2-PRD2 complex did not dissociate for the entire time of the experiment). The association of PRD1 with itself was very slow. However, the decay of the binding signal for the PRD1-PRD1 pair was also relatively slow, indicating that despite the low efficiency of PRD1 homodimerization, once the PRD1-PRD1 complex is formed it is stable.

As for the interaction between PRD1 and PRD2, similar results were obtained when PRD2 was injected over immobilized PRD1 and when PRD1 was passed over immobilized PRD2. The affinity between PRD1 and PRD2 is lower than the affinity of PRD2 for itself and higher than the affinity of PRD1 for itself. Although the dissociation of the PRD2-PRD2 complex was slower than the dissociation of the PRD1-PRD1 complex, indicating that the former is more stable than the latter, it is evident from the kinetic data that the PRD1-PRD2 complex is formed fast and is stable.

Taken together, the results obtained with the SPR demonstrate that PRD2 has a high affinity for itself and that the PRD2-PRD2 complex is very stable. PRD2 also has a high affinity for PRD1, and the PRD1-PRD2 complex is stable, albeit somewhat less than the PRD2-PRD2 complex. Formation of the PRD1-PRD1 complex is slow, but once it is formed, it is relatively stable. The hierarchy of the affinities between the BglG domains is PRD2-PRD2 > PRD1-PRD2 > PRD1-PRD1, but all of the three complexes are fairly stable once formed.

**DISCUSSION**

The BglG-like proteins are typically composed of an ~55-residue RNA-binding domain followed by two PRD domains, each ~100-residue long, that are very similar in their secondary structure and show some conservation in their amino acid sequence (9). These proteins bind to their RNA targets as dimers to accomplish antitermination (6, 25). The RNA-binding domain of BglG cannot mediate dimerization and antitermination in vivo (15). However, the structure of dimeric RNA-binding domains of two BglG homologues, SacY and LicT, from *B. subtilis* was studied by NMR and x-ray crystallography (12–14). These domains formed dimers at relatively high salt concentrations but at least for SacY it was reported that low-

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**Coomassie stain**

**Far-Western**

**Fig. 2.** Far-Western analysis of the interaction between the PRD domains of BglG. A, purified PRD1 and PRD2, each fused to MBP, and MBP alone were analyzed by SDS-PAGE and stained with Coomassie Blue. B, in A but the proteins were blotted onto nitrocellulose filters, probed with His-PRD1 (lanes 1–3), or His-PRD2 (lanes 4–6) and then with anti-His antibodies.

**Fig. 3.** SPR analysis of the interaction between the PRD domains of BglG. Purified His-tagged PRD1 or PRD2 (~1000 resonance units) were immobilized on Biacore sensorchips. In each sensorchip, flow cell 1 (Fc1) with no bound protein served as a reference. PRD2 (A) or PRD1 (B) was injected over the immobilized PRD1 and PRD2 at a concentration of 5 $\mu$m. This figure shows the subtracted (Fc1 – Fc3) sensorgrams that allowed direct visualization of specific binding. The association kinetics was followed for 2 min after the injection start (left arrow), and the dissociation kinetics was followed for 2–2.5 min after the injection stop (right arrow). C, the $K_D$ values were calculated by fit to a first order kinetic model based on measurements at various concentrations of the injected proteins.

| Chip-bound protein | Injected protein | $k_a$(M$^{-1}$s$^{-1}$) | $k_d$(s$^{-1}$) | $K_D$(M) |
|--------------------|-----------------|----------------------|----------------|---------|
| PRD2               | PRD2            | 5310                 | 1.19X10$^4$    | 2.2X10$^8$ |
| PRD1               | PRD2            | 4530                 | 1.91X10$^3$    | 4.2X10$^7$ |
| PRD2               | PRD1            | 4141                 | 1.36X10$^3$    | 3.3X10$^7$ |
| PRD1               | PRD1            | 1460                 | 1.34X10$^3$    | 9.1X10$^7$ |
ering the ionic strength resulted in dissociation of the dimers and that re-increasing the salt concentration did not result in refolding (12). Hence, the RNA-binding domain of the BglG-like antiterminators is not predicted to mediate their dimerization under physiological salt concentrations. Because the ability of these proteins to dimerize and bind to RNA is regulated by reversible phosphorylation at the PRD domains, it is predicted that the PRDs will mediate dimerization depending on their phosphorylation state and bring the two RNA-binding domains together. Indeed, the PRDs region of a LicT mutant crystallized as a homodimer (17). The results presented in this paper from one-hybrid, two-hybrid, Far-Western, and SPR analyses demonstrate that PRD2 of BglG mediates dimerization very efficiently as opposed to PRD1. Notably, although the rate of association of PRD1 with itself is very slow, the dissociation rate of the PRD1-PRD1 dimers is also slow, suggesting that once PRD1 homodimerizes, the complex is stable. This conclusion is supported by our findings that although PRD1 poorly mediated dimerization of the DNA-binding domain of xCI repressor, the resulting DBD-PRD1 fusion protein conferred immunity against λ infection, suggesting that the small number of dimers that were formed were stable. Therefore, we suggest that the process of BglG dimerization begins with the interaction of the two PRD2 domains that have a high affinity to each other. However, based on our results, when the PRD1 domains of the two monomers are brought together by the two PRD2 domains, they interact and remain bound. Hence, dimerization is predicted to propagate from the C to the N terminus, bringing the two RNA-binding domains together to enable RNA-binding. The slow association rate of PRD1 with itself, which is in marked contrast to the relatively high stability of the resulting homodimer, might suggest that the population of monomeric PRD1 is heterogeneous. The probability that a PRD1 monomer, which has a fold that favors the formation of homodimers, will find another monomer of its kind is low.

Our results showing that homodimerization of PRD2 is more efficient than homodimerization of the entire BglG protein, which contains both PRDs, suggests that PRD1 inhibits dimerization of a fraction of BglG population by folding on PRD2 and preventing PRD2-PRD2 interaction. This hypothesis is supported by our previously published result that a BglG derivative lacking the PRD1 domain antiterminated transcription better than wild-type BglG protein (15). The similarity between PRD1 and PRD2 in their primary, secondary, and tertiary structure and the flexible loop that connects them based on LicT structure (17) suggest that an interaction between PRD1 and PRD2 may occur in the cell. Therefore, we studied the interaction between PRD1 and PRD2 with various experimental approaches. The results presented here demonstrate that the affinity between PRD1 and PRD2 is high enough to allow their efficient interaction in vitro and in vivo. Preliminary cross-linking results from our laboratory demonstrate that PRD1 and PRD2 indeed interact intramolecularly in a fraction of cellular BglG monomers. Because intramolecular interaction between PRD1 and PRD2 and intermolecular interaction between BglG monomers are probably mutually exclusive, these results explain why PRD2 mediates dimerization better than the entire BglG protein. In addition, the existence of a monomeric form in which PRD1 and PRD2 are in close proximity might explain why the conserved Asp-100 and His-160, both in PRD1, are required for the phosphorylation of His-208 in PRD2 by BglF.

The activity of several BglG homologues, mainly from Gram-positive bacteria, e.g., LicT and SacT from B. subtilis, was shown to depend on functional enzyme I and HPr (26, 27). It was suggested that these antiterminators are positively con-

trolled by PEP-dependent enzyme I- and HPr-catalyzed phosphorylation. LicT was shown to be phosphorylated in vitro in the presence of the phosphorylated general PTS proteins on three of the four conserved histidines, i.e., the second conserved histidine in PRD1 and the two conserved histidines in PRD2 (28). Genetic analyses suggested that the two histidyl residues in PRD2 are important for LicT activation (28). In contrast, SacY from B. subtilis was shown to be active in the absence of functional enzyme I and HPr. Nevertheless, based on analysis of mutants, it was suggested that SacY is also multiply phosphorylated by enzyme I and HPr (29). The activity of the BglG-like antiterminators is subjected also to negative regulation exerted by their cognate sugar-specific sensors, enzymes II such as BglF, which phosphorylates them on one of their conserved histidines. The site on BglG, which is phosphorylated by BglF, was mapped to the first conserved histidine in PRD2 (10), although the conserved histidines in PRD1 are required for PRD2 phosphorylation. Interestingly, BglG lacks the second conserved histidine in PRD2. The site on which LevR, an activator from B. subtilis, is phosphorylated by its cognate enzyme II was also mapped to a conserved histidine in PRD2 (30). Thus, for BglG and LevR, the enzyme II-mediated inhibition process seems to rely on the presence of a negative charge on a conserved histidine in PRD2. In contrast, the site suggested for the phosphorylation of LicT and SacY by their respective enzymes II, BglP and SacX, is a conserved histidine in PRD1 (29, 31). Hence, the site on which the antiterminators are phosphorylated by their cognate enzymes II seems to be a conserved histidine in either PRD1 or PRD2. Taken together, the data that accumulated thus far suggest that the two consecutive homologous PRD domains have the potential to play similar roles in regulation. It has been suggested that the two PRDs arose from an ancestral gene duplication (32). During evolution, these two similar domains could have been shuffled and it seems that the actual PRD domain that is negatively phosphorylated is specific for the protein and the regulatory mechanisms to which it is subjected. It is plausible that the PRD, which gives tighter dimers, is the one to be negatively regulated. This will guarantee control of antitermination by phosphorylation and prevent phosphorylation from hampering antitermination. Indeed, a truncated LicT protein, which lacks PRD1, generated dimers (33), whereas a similar truncated BglG protein could not undergo dimerization of CI repressor DNA-binding domain and gave residual antitermination activity (15). Because LicT is negatively regulated by phosphorylation on PRD1 as opposed to BglG, these findings suggest that LicT-PRD1 mediates LicT dimerization, whereas dimerization of BglG is mediated by its PRD2 domain.

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