Phosphorylation of the Group A Streptococcal CovR Response Regulator Causes Dimerization and Promoter-Specific Recruitment by RNA Polymerase

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The group A streptococcus (GAS), Streptococcus pyogenes, is an important human pathogen that causes infections ranging in severity from self-limiting pharyngitis to severe invasive diseases that are associated with significant morbidity and mortality. The pathogenic effects of GAS are mediated by the expression of virulence factors, one of which is the hyaluronic acid capsule (encoded by genes in the has operon). The expression of these virulence factors is controlled by the CovR/S (CsrR/S) two-component regulatory system of GAS which regulates, directly or indirectly, the expression of about 15% of the genome. CovR is a member of the OmpR/PhoB family of transcriptional regulators. Here we show that phosphorylation by acetyl phosphate results in dimerization of CovR. Dimerization was not observed using a D53A mutant of CovR, indicating that D53 is the site of phosphorylation in CovR. Phosphorylation stimulated binding of CovR to a DNA fragment containing the promoter of the has operon (Phas) approximately twofold. Binding of CovR D53A mutant protein to Phas was indistinguishable from the binding of wild-type unphosphorylated CovR. In vitro transcription, using purified GAS RNA polymerase, showed that wild-type CovR repressed transcription, and repression was stimulated more than sixfold by phosphorylation. In the presence of RNA polymerase, binding at Phas of phosphorylated, but not unphosphorylated, CovR was stimulated about fourfold, which accounts for the difference in the effect of phosphorylation on repression versus DNA binding. Thus, regulation of Phas by CovR is direct, and the degree of repression of Phas is controlled by the phosphorylation of CovR.

The group A streptococcus (GAS; Streptococcus pyogenes) causes a wide range of illnesses in the human host. These range from mild, self-limiting infections such as pharyngitis and impetigo to severe, invasive infections such as streptococcal toxic shock syndrome and necrotizing fasciitis (3). In addition, diseases such as rheumatic fever, rheumatic heart disease, and acute glomerulonephritis can result from autoimmune responses following a streptococcal infection. The success of GAS in establishing and maintaining infection is dependent on the organism’s ability to adapt to various environments in the host. Therefore, an understanding of gene regulation in response to the host environment is necessary to develop methods to inhibit GAS pathogenesis.

Bacteria often sense and respond to the surrounding environment through the use of two-component regulatory systems (17, 25, 30). These systems typically employ a membrane-bound protein (sensor histidine kinase) that responds to an external environmental signal by autophosphorylation. This signal is then transduced to a cytoplasmic DNA binding protein (response regulator) via phosphorylation, resulting in activation or repression of specific target promoters. Response regulator proteins typically have two domains: a receiver domain that acts as the phosphoryl acceptor and a DNA binding domain whose affinity for DNA is stimulated by phosphorylation of the receiver domain (30). The two domains can be connected by linkers of various lengths (6). In GAS, the two-component regulatory system CovR/S (also called CsrR/S) controls the expression of approximately 15% of the genes (4, 11), including virulence factors (7, 15, 19) and genes involved in stress survival (4, 5). These genes are regulated either directly, with CovR/S acting primarily to repress gene expression, or indirectly, through the effect of CovR/S regulating other transcription factors.

One virulence factor whose transcription is repressed by CovR/S is the hyaluronic acid capsule (2, 7, 15, 19), synthesized by the proteins encoded by the has operon. The capsule, found on the outer surface of the GAS cell, delays phagocytosis and may be involved in initial colonization (18, 26, 27, 33). Previous work demonstrated that the response regulator, CovR, binds has promoter (Phas) DNA in vitro (8, 22), suggesting that CovR regulation is direct. As further evidence for this, disruption of four of the five CovR binding sites identified at Phas results in derepression of has transcription in vivo (8). However, it remained possible that CovR repression of Phas is indirect, for example by interfering with binding of an activator required for has transcription. In addition, CovR may not be sufficient for repression of Phas, since it may require a corepressor or cofactor expressed in the cell.

Phosphorylation of CovR appears to be important for regulation of Phas both in vitro and in vivo. In vitro, phosphorylated CovR (CovR-P) binds Phas DNA with a twofold greater affinity than that of unphosphorylated CovR (8). Alignment of the known phosphorylation domains of several response regulators homologous to CovR indicates that the aspartic acid at position 53 of CovR is most likely to be the residue phosphorylated (4). Using an in vivo reporter system for has transcription, Dalton and Scott (4) demonstrated that a CovR D53A...
mutant did not complement a CovR deletion strain for repression of Phas, whereas the wild-type allele expressed from the same plasmid did. This result suggests that phosphorylation of CovR is required for repression of Phas in vivo. However, whether the CovR D53A mutant protein is capable of being phosphorylated or binding DNA was not tested.

In this work, we investigated the effect of phosphorylation on the conformation of CovR and determined the effect of phosphorylation on DNA binding at Phas both in the presence and in the absence of RNA polymerase. We also purified a CovR D53A mutant protein and compared it to wild-type CovR for its ability to be phosphorylated and to bind DNA. Finally, we used an in vitro transcription system to determine whether CovR is sufficient to repress Phas and whether phosphorylation of CovR enhances this repression.

MATERIALS AND METHODS

Strains and media. Escherichia coli DH5α and XL1-Blue (Stratagene) were used for constructing all plasmids. E. coli LMG1914 (araC deletion strain) was used to purify CovR D53A mutant protein. All strains were grown in LB broth (28) with agitation at 30°C or 37°C. For cloning purposes ampicillin was used at 100 μg/ml. E. coli overexpressed in P. aeruginosa was grown in LB-glucose (28) with agitation at 30°C or 37°C. For cloning purposes ampicillin was used at 100 μg/ml.

Construction of the CovR D53A expression plasmid. The CovR gene from the chromosome of GAS strain JRS4 (29) was amplified using primers 5′-covR EcoRI (ccgccggaattc) and 3′-covR SphI (aag acatgcatgcCATATGACTTATTTCTCACG). Primer sets for the amplification were designed based on mismatches with JRS4, indicating the presence of differences in the CovR sequence of this strain. The resulting PCR product was cloned downstream of the PmBAD promoter between the EcoRI and SphI sites in pBAD30 (14) to construct pEU7545. To introduce an alanine substitution for D53 in the open reading frame of covR on this plasmid, primers D53A-S1 and D53A-A1 were used (4) following the QuickChange mutagenesis kit protocol (QIAGEN). The sequence of the resulting plasmid, pEU7550, was verified.

Purification of wild-type CovR and CovR D53A protein. Wild-type CovR was overexpressed in E. coli (13) and purified as described previously (8). For purification of CovR D53A protein, E. coli LMG1914 containing the CovR D53A expression plasmid (pEU7550) was grown at 37°C in LB with 30 μg/ml ampicillin to an optical density at 600 nm of 0.6. Expression of CovR D53A was induced by addition of 0.2% arabinose, and the culture was incubated for an additional 3 h at 30°C. Cells were lysed with a French press and pelleted, and CovR D53A inclusion bodies were solubilized in 6 M guanidinium HCl for 15 min. To remove the guanidinium HCl, the protein was dialyzed against equilibration buffer (50 mM Tris-Cl, pH 7.5, 50 mM NaCl, 2 mM DTT, 10% glycerol) at 4°C overnight. To remove additional contaminants, the protein was run through a HiTrap heparin column (Amersham Pharmacia Biotech), and the flowthrough fraction containing CovR D53A protein was collected. The protein was further purified through a HiTrap Q Sepharose column (Amersham Pharmacia Biotech) and eluted using a 30-ml 1 M NaCl gradient. The purified CovR D53A protein was then dialyzed against equilibration buffer (above).

Phosphorylation of CovR. To phosphorylate CovR, the protein was incubated for 2 h in 50 mM Tris-HCl, pH 7.4, 50 mM KCl, 20 mM MgCl2, and 25 mM acetyl phosphate. For in vitro transcriptions, following phosphorylation, acetyl phosphate was removed by purification through MicroBioSpin6 columns (BioRad). The protein concentration was determined using the Micro BCA protein assay kit (Pierce) standardized against bovine serum albumin.

Electrophoretic analysis. Protein samples were electrophoresed through a native nondenaturing 10% (w/v) polyacrylamide gel containing Tris-HCl (Bio-Rad) using 25 mM Tris, 250 mM glycine (pH 8.3) running buffer. The apparent molecular weight of CovR and CovR-P was determined using a Ferguson plot (9, 16). To construct a standard curve, α-lactalbumin, carbonic anhydrase, chicken egg albumin, and bovine serum albumin obtained from Sigma-Aldrich were subjected to electrophoresis on 8%, 9%, 10%, 11%, and 12% polyacrylamide gels as described above and the relative mobilities of the different protein species were determined. The slope of the line of relative mobility versus gel concentration was measured for each species, and the logarithm of these values was plotted versus the logarithm of the molecular weight. The apparent molecular weights of CovR and CovR-P were determined from the standard curve by interpolation.

RESULTS

Phosphorylation of CovR results in dimerization of the protein. Phosphorylation of purified CovR protein has been shown to enhance its DNA binding at several promoters, including Phas, Pska, PspER, PsaG, Psda, and Pcov (8, 13, 22). In addition, phosphorylated CovR enhances repression of transcription from Pcov and PsaG in vitro (10, 13). Although phosphorylation of purified His-tagged CovR was reported to result in multimerization of the protein in solution (22), gel filtration analysis did not define the higher-order species that resulted. To learn this, native CovR protein was purified as described previously (13) and incubated at 37°C with or without acetyl phosphate for 2 hours. The resulting species were analyzed on a gel under nondenaturing conditions (Fig. 1A). Following incubation with acetyl phosphate, most of the protein was converted to a single, slower-migrating species. To confirm this, a single phosphorylation group was confirmed by mass spectrometry (data not shown).

Interpolation of the molecular mass of CovR using a Ferguson plot (9, 16) (Fig. 1B) indicates that the unphosphorylated protein is ~33 kDa, close to the predicted molecular mass of monomeric CovR (26.7 kDa). The slower-migrating species visible after phosphorylation had an apparent molecular mass of ~60 kDa. Therefore, the phosphorylated form of the protein is likely to be a dimer of CovR.

The D53A residue of CovR is required for phosphorylation-dependent dimerization of the protein. Homology analysis predicts that phosphorylation occurs at the D53 residue of CovR (4). Therefore, we changed the aspartic acid at position 53 to alanine by site-directed mutagenesis and purified the native mutant protein. When CovR D53A protein was incubated for 2 hours with acetyl phosphate, its migration through a native gel was slightly slower than that of the unphosphorylated wild-type form, but there was no indication of a new species of the protein appearing upon phosphorylation (Fig. 1A). This indicates that the D53 residue of CovR is required for phosphorylation-dependent dimerization of the protein in vitro.

CovR D53A binds Phas DNA as efficiently as unphosphorylated, wild-type CovR. Introduction of an amino acid change in the phosphorylation domain of CovR might alter the structure of the protein and therefore affect its DNA binding domain. To test this, we performed DNase I protection analysis at Phas. At 3.1 μM, wild-type CovR protected the five previously defined regions of DNA at Phas, labeled CB-1 through CB-5.

Phosphorylation of CovR-Phas half-life. Following phosphorylation and removal of acetyl phosphate (see above), CovR-Phas was incubated at 37°C in the absence of acetyl phosphate and loaded at various time intervals on a native nondenaturing gel as described above. At each time interval, the fraction of CovR-Phas remaining was determined using ImageQuant software.

DNase I protection assay. Primers HAs5−130-S and HAs5+151 (8) were used to amplify a 277-bp segment of the JRS4 chromosome including the Phas region from −130 to +151 bp (with respect to the start of transcription). The sense primer in the PCR was radiolabeled as described previously (24), and DNase I protection assays were performed as described before (13).

In vitro transcription. GAS RNA polymerase and major sigma factor RpoD were purified as described previously (13). Plasmid pEU7020 (8), containing a region of the hsp promoter from −440 to +225 with respect to the transcriptional start, was used as the DNA template for runoff transcription reactions. Reactions were performed as described previously (10).
The mutant CovR D53A protein also protected these five regions of DNA at the same concentration (3.1 μM protein; Fig. 2). Therefore, the DNA binding of CovR D53A appears to be unaffected by the mutation in its phosphorylation domain. Because the mutant protein has retained its DNA binding ability, it appears that the conformation of the protein has not been grossly altered by the substitution of A for D53. Phosphorylation of wild-type CovR enhanced the binding affinity to Phas by approximately twofold (Fig. 2), in agreement with previous results obtained using gel mobility shift assays (8). In contrast, the binding affinity of CovR D53A for Phas DNA showed no change following incubation with acetyl phosphate (Fig. 2). These results indicate that the aspartate at position 53 of CovR is required for the enhanced DNA binding that is seen following phosphorylation of the protein.

Phosphorylated CovR specifically represses has transcription in vitro. We used an in vitro GAS transcription system (13) to determine whether CovR is sufficient to repress Phas

FIG. 1. Phosphorylation of CovR by acetyl phosphate (AP). A. Purified CovR and CovR D53A were incubated with or without acetyl phosphate for 2 hours prior to being loaded on a native 10% (wt/vol) polyacrylamide gel under nondenaturing conditions. B. Ferguson plot analysis depicting the apparent molecular mass of CovR (square) and CovR-P (triangle) using a standard curve (circles); see Materials and Methods.

FIG. 2. DNase I footprint of CovR and CovR D53A protein incubated with or without acetyl phosphate (as indicated) at the sense strand of Phas (from −130 to +151). The concentrations of protein used (CovR or D53A) are shown at the top of the image. Protected regions are indicated by vertical lines, and promoter elements (−35 and −10) as well as CovR binding sites (CB-1 through CB-5) are labeled. The bent arrow indicates the start of has transcription (23).
and to assess the effect of phosphorylation of CovR on repression. Because acetyl phosphate interferes with transcription reactions (data not shown), CovR-P was purified through desalting columns following incubation with the phosphoryl donor (see Materials and Methods). We determined the CovR-P half-life to be 83 min following the removal of acetyl phosphate (Fig. 3). Therefore, the protein likely remained phosphorylated for the duration of the transcription experiments.

The DNA template used, pEU7020 (8), contains a 665-bp region that includes Phas. This plasmid was linearized at a restriction site downstream of the Phas start of transcription with either NotI or PstI (Fig. 4). In vitro transcription reactions produced three transcripts originating from this 665-bp region. The size of the transcript labeled has was consistent with that predicted for a transcript originating from the has promoter: 260 bp from the NotI-digested template and 245 bp from the PstI-digested template. The other two transcripts, C1 and C2, were not previously identified in vivo, nor did we identify these transcripts by primer extension analysis of RNA isolated from CovR⁺ or CovR⁻ cells (data not shown). Because we do not know whether these transcripts are produced in vivo, we are unable to speculate on whether they are involved in expression of the has operon. However, neither of these transcripts was subject to regulation by CovR in vitro, so they were not analyzed further.

To evaluate repression, increasing concentrations of CovR or CovR-P were incubated with the DNA prior to addition of RNA polymerase (Fig. 5). As an internal transcript control, pEU7020 was digested at a site (XcmI) downstream of the promoter for the spectinomycin resistance marker (Paad), resulting in an additional transcription product of 510 bp (Fig. 5). The results show that CovR-P specifically repressed transcription from Phas at lower concentrations than did unphosphorylated CovR. At 0.08 μM CovR-P, the relative amount of has transcript was reduced by 50% (Fig. 5B). In contrast, 0.52 μM unphosphorylated CovR was required to reduce has transcription by 50%, a 6.5-fold difference in concentration. These results indicate that CovR repression of Phas is direct and that no other factor is required for repression. In addition, these results show that CovR repression is enhanced by phosphorylation.

Because DNA supercoiling can influence the effect of a DNA binding protein on transcription regulation, we performed these same repression experiments using supercoiled DNA.
template and obtained similar results (data not shown). Therefore, the linear or supercoiled state of the DNA template did not affect CovR repression in vitro.

Stimulation of CovR-P binding to Phas by RNA polymerase.

To explain the difference in the stimulation of DNA binding (twofold) and repression of transcription from Phas (6.5-fold) by phosphorylation of CovR, we repeated DNA binding experiments in the presence of RNA polymerase using the same conditions as those employed in the in vitro transcription reactions. In the presence of RNA polymerase, unphosphorylated CovR bound to sites CB-1 and CB-2 at 3 μM CovR (Fig. 6), the same concentration at which protection was observed in the absence of RNA polymerase (Fig. 2). Thus, RNA polymerase has no effect on the binding of unphosphorylated CovR. In contrast, in the presence of RNA polymerase, CovR-P bound to sites CB-1 and CB-2 at 0.4 μM CovR-P (Fig. 6), rather than at 1.6 μM CovR-P in the absence of RNA polymerase (Fig. 2). Thus, RNA polymerase stimulates binding of CovR-P to Phas fourfold. The stimulation occurs at the expected molar ratio of 10 of CovR-P (0.4 μM) to RNA polymerase (0.04 μM), since at Phas CovR-P binds as a dimer at five sites (8) and RNA polymerase at one. The combination of phosphorylation and the presence of RNA polymerase together results in a seven- to eightfold increase in the affinity of CovR for Phas. This degree of stimulation is in accord with the 6.5-

FIG. 6. DNase I footprint of CovR and CovR-P on the antisense strand of Phas (from −130 to +151) in the presence of 0.045 μM RNA polymerase. Protected regions are indicated by vertical lines, and promoter elements (−35 and −10) as well as CovR binding sites (CB-1 through CB-5) are labeled. Lanes 1 to 4, DNA sequence ladder. Lanes 5 to 10, 0, 0.38, 0.75, 1.5, 3, and 6 μM CovR, respectively. Lanes 11 to 15, 0, 0.11, 0.21, 0.42, and 0.83 μM CovR-P, respectively. The sequence ladder is cropped from a longer exposure of the gel.
fold stimulation of repression seen in the in vitro transcription experiment (Fig. 5).

**DISCUSSION**

**Phosphorylation of CovR causes dimerization.** We have found that upon phosphorylation by the low-molecular-weight phosphoryl donor acetyl phosphate, CovR forms a single species with an altered electrophoretic mobility characteristic of a dimer (Fig. 1). This response to phosphorylation is similar to that of other members of the OmpR family of response regulator proteins (1, 20, 23, 31, 32). Production of the dimeric species of CovR requires the residue D53 since the mobility of CovR carrying a D53A mutation is unaffected by incubation with acetyl phosphate. Based on sequence and structural comparisons of CovR with other members of the OmpR family, this is the residue that would be expected to act as the phosphoryl acceptor (4). Structural analysis of the receiver domains of OmpR family members showed that phosphorylation (mimicked by incubation of the protein with beryllium fluoride) causes a structural transition in the protein that exposes a previously hidden dimerization interface known as the α4-β5-α5 interface (31). Electrostatic and hydrophobic interactions using this interface occur between a series of conserved residues in each monomer, resulting in the formation of a dimer with twofold rotational symmetry. Since these critical residues are conserved in CovR, it seems that the response of CovR to phosphorylation is similar to, and occurs by the same mechanism as, other members of the OmpR family.

**Phosphorylation of CovR increases DNA binding affinity.** Using DNA footprinting techniques, we have shown that phosphorylation of CovR results in a modest (twofold) increase in DNA binding affinity at Phas (Fig. 2). This is the same increase that we previously observed in electrophoretic gel mobility shift assays (8). This compares to a fourfold increase in binding affinity at Psag (13) and a greater-than-eightfold increase in binding affinity at Psag (10). Although the precise basis for the different increases in binding affinity at these promoters upon phosphorylation of CovR remains unclear, differences in the positions and orientations of CovR binding sites and in the conformations that unphosphorylated and phosphorylated CovR can adopt must be important.

**Phosphorylation of CovR stimulates repression of Phas.** In an in vitro system containing purified GAS RNA polymerase, CovR represses Phas transcription, and this repression is enhanced approximately sixfold by prior phosphorylation of CovR (Fig. 5). Thus, CovR acts directly in the absence of other factors to repress transcription from Phas. In vivo, CovR is required for repression of transcription from Phas. We previously suggested that this repression requires phosphorylation of CovR since transcription from Phas is constitutive in a mutant expressing CovR D53A (4). In support of this, we have shown here that the binding of CovR D53A is indistinguishable from binding of unphosphorylated CovR (Fig. 2), so the CovR protein is not grossly disrupted by the mutation. Furthermore, we find a significant stimulation (6.5-fold) of phosphorylation of CovR on repression of Phas, as measured by in vitro transcription experiments (Fig. 5).

**RNA polymerase stimulates CovR-P binding and repression at Phas.** The stimulation of transcriptional repression by phosphorylation of CovR (6.5-fold) was significantly greater than the stimulation of DNA binding affinity (twofold) at Phas in experiments using similar linear DNA molecules. We found that this difference in transcriptional repression and DNA binding is accounted for by stimulation of CovR-P binding by RNA polymerase. In contrast, at Psag and at Psag, the stimulation of repression and that of DNA binding were similar (~3-fold at Psag and ~8-fold at Psagag (10, 13). Thus, RNA polymerase specifically stimulates the binding of CovR-P to Phas but not to Psag or Psag.

Because the stimulation by RNA polymerase of CovR-P binding is promoter specific, it is unlikely that these proteins associate in the absence of DNA, as RNA polymerase does with MarA and SoxS of E. coli (12, 21). Instead, it appears that interactions between CovR-P and RNA polymerase occur only in the presence of DNA with the appropriate sequence.

Extended regions of DNA bound by CovR contain AT TARA sequences that have been shown to be required for binding (8, 10, 13). In addition, CovR also binds other related sequences at Psag (13) and Psag (10). There are over 4,000 ATTARA potential CovR binding sites in the GAS strain SF370 genome, and many of these occur within open reading frames. Therefore, an additional mechanism is likely to be required to direct CovR to bind preferentially at regulatory regions. If the DNA sequence is appropriate, as at Psag (10), cooperative interactions between individual CovR-P molecules will increase the affinity for the promoter. At other promoter regions, such as Phas, where there is no interaction between CovR-P molecules bound at different sites (8), interactions between CovR-P and RNA polymerase, rather than between CovR-P molecules alone, may occur to facilitate CovR-P binding. Thus, we propose that recruitment of CovR-P to promoters can be accomplished by either of two mechanisms (or a combination of both), depending upon the DNA sequence of the promoter.

Further work is required to understand the way in which the pattern of CovR binding sites at specific promoters influences these protein-protein interactions and the effect of these interactions on the hierarchy of promoters repressed by CovR in vivo.

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