Intracellular autoregulation of the Mycobacterium tuberculosis PrrA response regulator

Fanny Ewann, Camille Locht and Philip Supply

INTRODUCTION

Two-component systems are major regulatory systems for bacterial adaptation to environmental changes. During the infectious cycle of Mycobacterium tuberculosis, adaptation to an intracellular environment is critical for multiplication and survival of the micro-organism within the host. The M. tuberculosis prrA gene, encoding the regulator of the two-component system PrrA–PrrB, has been shown to be induced upon macrophage phagocytosis and to be transiently required for the early stages of macrophage infection. In order to study the mechanisms of regulation of the PrrA–PrrB two-component system, PrrA and the cytoplasmic part of the PrrB histidine kinase were produced and purified as hexahistidine-tagged recombinant proteins. Electrophoretic mobility shift assays indicated that PrrA specifically binds to the promoter of its own operon, with increased affinity upon phosphorylation. Moreover, induction of fluorescence was observed after phagocytosis of a wild-type M. tuberculosis strain containing the gfp reporter gene under the control of the prrA–prrB promoter, while this induction was not seen in a prrA/B mutant strain containing the same construct. These results indicate that the early intracellular induction of prrA depends on the autoregulation of this two-component system.

Mycobacterium tuberculosis possesses a complex infectious cycle, which includes intra- and extra-cellular phases, both within and outside the lungs, as well as a latency phase, suggesting that the expression of many of its genes must be subjected to regulation. However, the regulatory mechanisms governing the adaptive responses of M. tuberculosis, especially during phagocytosis, are still poorly understood. The M. tuberculosis genome contains 11 pairs of genes encoding two-component systems, in addition to a few isolated genes encoding orphan histidine kinases or response regulators (Cole et al., 1998). Several of these genes have been characterized at least partially (Dasgupta et al., 2000; Ewann et al., 2002; Graham & Clark-Curtiss, 1999; Haydel et al., 1999; Himpens et al., 2000; Perez et al., 2001; Sherman et al., 2001; Supply et al., 1997; Via et al., 1996; Zahrt & Deretic, 2000, 2001). For example, an M. tuberculosis strain with a mutation in the phoP gene was found to be impaired in intra-cellular growth within macrophages, one of the major target cells of M. tuberculosis, and its virulence was found to be attenuated in mice (Perez et al., 2001). MprA–MprB is required for persistence in murine infection (Zahrt & Deretic, 2001) and DevR–DevS is induced in response to hypoxia and required to survive it (Boon & Dick, 2002; Park et al., 2003; Sherman et al., 2001). MtrA–MtrB was found to be essential for survival, as so far it has not been possible to obtain mtrA knockout strains of M. tuberculosis (Zahrt & Deretic, 2000). In addition, mtrA has been shown to be upregulated upon phagocytosis in Mycobacterium bovis BCG but not in M. tuberculosis (Via et al., 1996). The upregulating mechanism in M. bovis BCG has not yet been identified. The PrrA–PrrB system has been found to be induced after macrophage phagocytosis and to be transiently required during the early stages of the macrophage infection (Ewann et al., 2002; Graham & Clark-Curtiss, 1999). The PrrA–PrrB system belongs to a wide subfamily of two-component systems, of which OmpR–EnvZ is the prototype. Many members of this subfamily have been demonstrated to be autoregulated. However, autoregulation is not a general rule, as illustrated by the hilA gene in Salmonella typhimurium (Bajaj et al., 1996; Lucas et al., 1996; Via et al., 1999).  

Abbreviation: His6, hexahistidine.
et al., 2000). Here, we investigated the possible autoregulation of prrA and its role in the induction of the expression of this gene upon phagocytosis of *M. tuberculosis*.

**METHODS**

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1. All cloning steps were carried out in *Escherichia coli* XL-1 Blue (Stratagene). Recombinant hexahistidine (His₆)-PrrA and His₆-PrrB were produced in *E. coli* M15 (Qiagen) and SG13009 (Qiagen), respectively. All DNA fragments were amplified by PCR from chromosomal DNA of the *M. tuberculosis* clinical isolate Mt103 (Jackson et al., 1999). The *M. tuberculosis* Mt21D3 mutant derivative, which contains a transposon inserted five nucleotides upstream of the predicted *prrA* start codon, has been described previously. This insertion presumably prevents transcription of the *prrA* operon, as a transcriptional terminator is present at the 3' end of the *aph* gene in the transposon and results in an impairment of the intracellular multiplication capacity during the first days of murine macrophage infection (Ewann et al., 2002). This strain will be subsequently referred to as the prrA/B mutant.

pflu1Sm was constructed by inserting a streptomycin-resistance cassette into pflu1 (Ewann et al., 2002) digested with EcoRV. This cassette was obtained by digesting pHP450 (Prentki & Krisch, 1984) with HindIII and Klenow fragment (Roche Diagnostic).

PrrA and the cytoplasmic domain of PrrB were produced as His₆-tagged recombinant proteins using pQE-30 (Qiagen). The *prrA* coding sequence was amplified by PCR using the oligonucleotide pair 5'-AAAAAGATCTATGGGCGGCATGGACACTGGTGTGA-3' and 5'-AAAAAGCTTTATCTGATACCCGACGACATCCGACTGCAC-3', and primers include a BglII and a HindIII restriction site, respectively (underlined). The cytoplasmic domain of PrrB was identified by alignment against the EnvZ and SenX3 sequences (Forst et al., 1989; Himpens et al., 2000) as the C-terminal part of the protein starting from amino acid 204. The corresponding DNA sequence was amplified by PCR using oligonucleotides 5'-GGGATCCATCGAGATCCTGCAGGACACATCCGACTGCAC-3' and 5'-TITTAAGCTTACTGGTCCCAGGAAAGGC-3', containing a BamHI and a HindIII restriction site, respectively (underlined). The amplified fragments were digested with BamHI and HindIII and inserted into pQEs005 restricted by the same enzymes to yield pQEs00-PrrA and pQEs00-PrrB, respectively.

**Purification of the His₆-tagged recombinant proteins under native conditions.** The recombinant *E. coli* strains containing pQE-PrrA and pQE-PrrB were grown in 1 L LB medium containing 100 μg ampicillin ml⁻¹ and 25 μg kanamycin ml⁻¹. When the OD₆₀₀ value reached 1.2–1.4, the expression of the genes encoding the recombinant proteins was induced with 1 mM IPTG for 3 h. The cells were then harvested by centrifugation, resuspended in 5 ml lysis buffer (300 mM NaCl, 50 mM Na₂HPO₄, 10 mM imidazole, pH 8) per gram of fresh weight. The cells were then lysed using a French Press under a pressure of 1000 p.s.i. (6.9 MPa). The lysates were clarified by centrifugation at 10,000 g for 20 min. The supernatants were filtered using a 0.45 μm filter before loading onto a 1.5 ml Ni-NTA column (Qiagen) equilibrated in lysis buffer. The column was washed first with lysis buffer until the ODₓ₀₀ value reached less than 0.01 and then with 5 ml lysis buffer containing 50 mM imidazole. The proteins were eluted with 7 ml lysis buffer containing 250 mM imidazole, and fractions of 1.5 ml were collected. The fractions were analysed by SDS-PAGE, using a 12.5% polyacrylamide gel, and Coomassie blue staining. After dialysis overnight against PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 150 mM NaCl, 1 mM EDTA, pH 8), the proteins were transferred to nitrocellulose membranes and blotted with a 1/5000 dilution of a mouse monoclonal antibody to His₆ (Invitrogen). After incubation with a 1/1000 dilution of a goat anti-mouse IgG (H+L) conjugated to horseradish peroxidase (Amersham), the membranes were incubated with substrate solution (0.005% diaminobenzidine, 0.005% H₂O₂ in 10 mM Tris-HCl, pH 7.5) for 5 min. The gels were continuously monitored until the background was reduced to an acceptable level. Both His₆-PrrA and His₆-PrrB were purified as expected from these experiments.

**Table 1.** Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><em>M. tuberculosis</em> Mt103</td>
<td>Clinical isolate</td>
<td>Jackson et al. (1999)</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> Mt21D3</td>
<td>prrA-deficient Mt103 derivative</td>
<td>Ewann et al. (2002)</td>
</tr>
<tr>
<td><em>M. bovis</em> BCG Pasteur 1173P2</td>
<td>Vaccine strain</td>
<td>World Health Organization, Stockholm (Sweden)</td>
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<tr>
<td><em>E. coli</em> XL-1 Blue</td>
<td>supE44 hsdRI71 recA1 endA1 gyrA46 thi reIAl Lac⁺ F⁻ [proAB⁺ lacB⁺ lacZAM15 Tn10 (Tet⁺)]</td>
<td>Stratagene</td>
</tr>
<tr>
<td><em>E. coli</em> M15(pREP4)</td>
<td>Na⁺ Str⁺ Rif⁺ Lac⁺ Ara⁻ Gal⁻ Mtl⁺ F⁻ RecA⁺ Uvr⁺ Lon⁺ (lacI Kan⁺)</td>
<td>Qiagen</td>
</tr>
<tr>
<td><em>E. coli</em> SG13009(pREP4)</td>
<td>Na⁺ Str⁺ Rif⁺ Lac⁺ Ara⁻ Gal⁻ Mtl⁺ F⁻ RecA⁺ Uvr⁺ Lon⁺ (lacI Kan⁺)</td>
<td>Qiagen</td>
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<tr>
<td>Plasmid</td>
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<td>pZero-2</td>
<td><em>E. coli</em> cloning vector</td>
<td>Invitrogen</td>
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<tr>
<td>pCR3-TOPO</td>
<td><em>E. coli</em> cloning vector</td>
<td>Invitrogen</td>
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<td>pQE30</td>
<td><em>E. coli</em> plasmid for production of His₆-tagged protein</td>
<td>Qiagen</td>
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<td>pQE30-PrrA</td>
<td><em>E. coli</em> plasmid for production of His₆-PrrA</td>
<td>This study</td>
</tr>
<tr>
<td>pQE30-PrrB</td>
<td><em>E. coli</em> plasmid for production of His₆-PrrB</td>
<td>This study</td>
</tr>
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<td>pJFX4</td>
<td><em>E. coli</em>-mycobacterial shuttle vector containing a constitutive promoter upstream of gfp; Km⁺</td>
<td>Triccas et al. (1999)</td>
</tr>
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<td>pFluo1</td>
<td><em>E. coli</em>-mycobacterial shuttle vector containing the prrA promoter upstream of gfp; Km⁺</td>
<td>Ewann et al. (2002)</td>
</tr>
<tr>
<td>pHP450</td>
<td><em>E. coli</em> plasmid; Sm⁺</td>
<td>Prentki &amp; Krisch (1984)</td>
</tr>
<tr>
<td>pFluo1Sm</td>
<td>pFluo1; Km⁺, Sm⁺</td>
<td>This study</td>
</tr>
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2 mM KHPO₄ containing 10% (v/v) glycerol, the protein concentrations were measured using BCA kit (Pierce); the proteins were dispensed into aliquots and then stored at ~20°C.

**Phosphorylation assays.** Phosphorylation assays were performed using 2 µg of His₆–PrrB incubated for 20 min at 37°C in the presence of 10 µCi of [γ⁻³²P]ATP (3000 Ci mmol⁻¹, 111 TBq mmol⁻¹; Amersham Biosciences) in 20 µl of phosphorylation buffer containing 100 mM Tris/HC1 (pH 8.0), 50 mM KCl and 5 mM MnCl₂. For phosphorylation assays, 20 µl of phosphorylation buffer containing 10 µg of His₆–PrrA were subsequently added. The reactions were stopped by the addition of 5 µl of 0.5 M EDTA (pH 8.0), and the incubation mixtures were subjected to SDS-PAGE using a 12:5% polyacrylamide gel. After electrophoresis and Coomassie blue staining, the gel was dried and exposed for autoradiography to an X-ray film (Biomax; Kodak).

**Electrophoretic mobility shift assays.** The promoter region of the prrA–prrB operon, PprrA/B, was amplified by PCR using primers prrA/FF (5’-TCGGGGATTGTCGACACCAYC-3’) and prrA/R (5’-CCATTGCGTGATTACCGTC-3’). The amplified fragment containing the entire intergenic region separating prrA from its flanking gene (Rv0904) was sequenced and then labelled by T4 kinase (Roche diagnostics) using 10 µCi of [γ⁻³²P]ATP (3000 Ci mmol⁻¹; Amersham Biosciences). One fragment of the labelled PCR fragment was incubated for 20 min at room temperature with 0–2 µg of His₆–PrrA, in 10 µl binding buffer containing 2 mM Tris/HC1 (pH 8.0), 0.4 mM MgCl₂, 10 mM KCl, 200 µM DTT, 10% (v/v) glycerol and 0.01% Nonidet P40. The reaction mixtures were loaded onto a 12% (w/v) polyacrylamide/45 mM Tris-borate/1 mM EDTA (pH 8.0) native gel and subjected to electrophoresis. After drying, the gel was exposed to an X-ray film. When the effect of phosphorylation on binding was tested, His₆–PrrA was phosphorylated prior to the assay in the same phosphorylation buffer as above containing 0.5 mM ATP instead of radiolabelled ATP.

**Bone-marrow macrophage infection and flow cytometry analysis.** Murine bone-marrow-derived macrophages were prepared and grown as described previously (Ewann et al., 2002). The infection assays were performed with 2 × 10⁵ cells per well in 24-well cell culture clusters (Techno Plastic products). After removing the culture medium, 1 ml of a suspension of the M. tuberculosis M103 wild-type or of the Mtb21D3 prrA/B mutant strain (Ewann et al., 2002), each containing pHluor1Sm, or of BCG containing pHluor1Sm was added to obtain an m.o.i. of 10:1. Control wells containing non-infected macrophages were filled with 1 ml fresh culture medium. After 4 h incubation at 37°C, the cells were washed three times in PBS to remove extracellular bacteria. The cells were then scraped and resuspended in 300 µl PBS; the fluorescence was analysed using a FACS Vantage apparatus (BD Bioscience). The non-infected macrophage suspension was used as a reference to define the macrophage cell population, to exclude free bacilli and to eliminate the effects of macrophage autofluorescence.

**RESULTS**

**Production of the recombinant proteins under non-denaturing conditions**

The PrrA regulator and the cytoplasmic domain of the PrrB sensor were produced as recombinant His₆–tagged proteins and purified under non-denaturing conditions to preserve enzymic activity. Since the amino-terminal transmembrane regions are dispensable for in vitro activity of most histidine kinases, this region of PrrB was not included to avoid solubility problems. A major protein with an apparent molecular mass of 30 kDa was detected in the soluble fraction of IPTG-induced E. coli M15(pQE-PrrA) upon SDS-PAGE and Coomassie blue staining (Fig. 1a, lane 2). This protein was then purified by Ni-NTA affinity chromatography (lane 3). In the soluble fraction of IPTG-induced E. coli SG13009(pQE30-PrrB), no major band could be detected in the range of the expected size for His₆–PrrB (Fig. 1b). Nevertheless, a protein with an apparent molecular mass of 31 kDa could be purified after Ni-NTA chromatography from this fraction. The observed molecular masses of the two recombinant proteins are slightly higher than expected for His₆–PrrA and His₆–PrrB (27.0 and 27.3 kDa, respectively), as frequently observed for His₆–tagged proteins. The identity of the purified His₆–tagged proteins was confirmed by immunoblotting using anti-His tag antibodies (not shown).

**Phosphotransfer between His₆–PrrB and His₆–PrrA**

The His₆–PrrB and His₆–PrrA phosphorylation assays were performed in the presence of Mn²⁺ ions, which have been demonstrated to be more efficient than Mg²⁺ ions for the mycobacterial TrcS histidine kinase activity (Haydel et al., 1999). As shown in Fig. 2, His₆–PrrB was able to auto-phosphorylate (lane 1). When His₆–PrrA was added to the His₆–PrrB phosphorylation mixture, phosphotransfer to His₆–PrrA was observed (lane 2). In the absence of His₆–PrrB, no detectable His₆–PrrA phosphorylation occurred (lane 3).

**His₆–PrrA DNA binding activity**

The ability of His₆–PrrA to bind to a 317 bp PCR fragment containing the prrA–prrB promoter region was assessed by
electrophoretic mobility shift assays. Fig. 3 shows that His6–PrrA was able to bind to this region. Binding to the labelled target was inhibited by the presence of an excess of unlabelled specific competitor (lane 4) but not by an excess of non-specific DNA (lane 5). These results indicate that His6–PrrA specifically binds to the promoter region and suggest that the genes are autoregulated.

To test the effect of phosphorylation on the His6–PrrA binding activity, mobility shift assays were carried out using the same DNA target and phosphorylated His6–PrrA. The electrophoretic mobility shifts were increased with phosphorylated His6–PrrA in comparison to unphosphorylated His6–PrrA ~ P were compared with each other (compare lanes 2 and 3 with lanes 7 and 8, respectively).

**Autoregulation of the prrA–prrB operon**

To confirm autoregulation of the prrA–prrB operon, we introduced pFluo1Sm, a plasmid containing the gfp reporter gene under control of the prrA–prrB promoter region, into the M. tuberculosis Mt103 wild-type strain or the Mtb21D3 prrA/B mutant derivative (Ewann et al., 2002). Since prrA–prrB is not expressed in axenic culture conditions, and since the expression of this operon is induced early after macrophage phagocytosis (Graham & Clark-Curtiss, 1999; Ewann et al., 2002), murine bone-marrow-derived macrophages were infected with the recombinant strains. A BCG strain containing the same construct and a BCG strain containing pJFX4 and constitutively expressing gfp were used as controls. The fluorescence of the infected macrophages was measured by flow cytometry. Induction of fluorescence was readily observed with macrophages infected by BCG or M. tuberculosis Mt103 containing the prrA::gfp construct (Fig. 4b, c). In contrast, no fluorescence was detected when the macrophages were infected with the M. tuberculosis prrA/B mutant containing the prrA::gfp construct (Fig. 4d). Although effects of different copy numbers of the prrA::gfp plasmid in the wild-type and in the prrA/B mutant can not be totally ruled out, these results, taken together with the results of the mobility shift assays, indicate that the prrA–prrB operon is autoregulated in M. tuberculosis.

**DISCUSSION**

Adaptive responses upon macrophage infection are probably critical in the infectious cycle of the intracellular pathogen M. tuberculosis. The genes of at least three two-component systems have been shown to be induced in M. tuberculosis or in M. bovis BCG during growth within macrophages (Ewann et al., 2002; Graham & Clark-Curtiss, 1999; Zahrt & Deretic, 2001). Among these systems, the PrrA–PrrB system has been shown to be transiently required for early intracellular multiplication of M. tuberculosis (Ewann et al., 2002). Electrophoretic mobility shift assays indicate that PrrA specifically binds to its own promoter region, suggesting that, like for several other response regulators belonging to the OmpR family, the prrA gene expression is autoregulated. The absence of detectable prrA expression in culture growth conditions hindered a more detailed mapping of the prrA transcriptional start site using primer extension experiments. Recent studies using electrophoretic mobility shift assays and reporter gene expression in heterologous E. coli or M. smegmatis systems have...
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suggested that the RegX3 and TrcR mycobacterial response regulators are also autoregulated (Haydel et al., 2002; Himpens et al., 2000). However, the signals involved in this autogenous control have not been identified yet. Here, autoregulation of the PrrA–PrrB system was indicated by the fact that intracellular expression of prrA::gfp depends on the presence of the PrrA–PrrB system in M. tuberculosis itself, as it is abolished in a M. tuberculosis prrA/B mutant. Conversely, this abolition shows that this autoregulatory loop controls the intracellular activation of prrA–prrB.

Increased binding to the prrA–prrB promoter region was observed upon phosphorylation of PrrA, which is consistent with the fact that the intracellular activation of the prrA–prrB operon actually depends on both the presence and activity of the PrrA–PrrB two-component system itself. The in vitro transphosphorylation was relatively inefficient under the conditions used in this study, as only a minor fraction of PrrA could be phosphorylated via PrrB. Therefore, the observed effect of PrrA phosphorylation on DNA binding was probably not optimal. Modifications of the Mn²⁺ concentrations or the replacement of Mn²⁺ by other bivalent ions did not significantly improve the enzymic activities (data not shown). It may be possible that optimal phosphotransfer requires additional factors yet to be identified or requires PrrB sequences that are absent from His₆–PrrB.

Several other M. tuberculosis genes have been shown to be induced concomitantly to prrA during the first days of the macrophage infection (Graham & Clark-Curtiss, 1999). These genes encode proteins with various functions, such as sigma factors and cation transporters, as well as proteins involved in lipid and cell-wall metabolism or in intracellular invasion or persistence in mice (Chitale et al., 2001; Cole et al., 1998; Kolattukudy et al., 1997; Manganelli et al., 1999; McKinney et al., 2000). The potential control of intracellular induction of these genes by the PrrA–PrrB system can now

Fig. 4. Autoinduction of prrA after macrophage infection. Mean fluorescence intensities of murine bone-marrow–derived macrophages were analysed after infection by recombinant BCG (b), M. tuberculosis Mt103 (c) or the prrA/B-deficient strain Mt21D3 (d) containing prrA::gfp, as described in Methods. Non-infected macrophages (a) were used as a negative control.
be investigated using a strategy similar to that described here and used to demonstrate prrA autoregulation. This approach may perhaps be complemented by a non-targeted, albeit more delicate, proteomic and transcriptomic analysis of the available prrA/B mutant grown intracellularly.

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