The Ser/Thr Protein Kinase PknB Is Essential for Sustaining Mycobacterial Growth

Pablo Fernandez,1 Brigitte Saint-Joanis,2 Nathalie Barilone,3 Mary Jackson,3 Brigitte Gicquel,3 Stewart T. Cole,2 and Pedro M. Alzari1*

Unité de Biochimie Structurale (URA CNRS 2185),1 Unité de Génétique Moléculaire Bactérienne,2 and Unité de Génétique Mycobactérienne,3 Institut Pasteur, 25/28 rue du Docteur Roux, 75724 Paris, France

Received 3 July 2006/Accepted 5 September 2006

The receptor-like protein kinase PknB from Mycobacterium tuberculosis is encoded by the distal gene in a highly conserved operon, present in all actinobacteria, that may control cell shape and cell division. Genes coding for a PknB-like protein kinase are also found in many more distantly related gram-positive bacteria. Here, we report that the pknB gene can be disrupted by allelic replacement in M. tuberculosis and the saprophyte Mycobacterium smegmatis only in the presence of a second functional copy of the gene. We also demonstrate that eukaryotic Ser/Thr protein kinase inhibitors, which inactivate PknB in vitro with a 50% inhibitory concentration in the submicromolar range, are able to kill M. tuberculosis H37Rv, M. smegmatis mc²155, and Mycobacterium aurum A+ with MICs in the micromolar range. Furthermore, significantly higher concentrations of these compounds are required to inhibit growth of M. smegmatis strains overexpressing PknB, suggesting that this protein kinase is the molecular target. These findings demonstrate that the Ser/Thr protein kinase PknB is essential for sustaining mycobacterial growth and support the development of protein kinase inhibitors as new potential antituberculosis drugs.

Mycobacterium tuberculosis is the causative agent of tuberculosis, a major world health problem that is responsible for the deaths of over two million people every year. A better understanding of the biology of the tubercle bacillus, with the goal of unveiling and validating new therapeutic targets, is an imperative requirement for improving the control and treatment of tuberculosis. The study of signaling elements, in particular Ser/Thr protein kinase systems (STPKs), is of outstanding interest in this context, given their likely important roles in mycobacterial physiology and virulence, as well as the available expertise on the design of specific inhibitors for eukaryotic STPKs, which currently represent one of the most actively studied groups of drug targets.

Signal transduction in prokaryotes is conducted primarily by two-component regulatory systems, basically consisting of a sensor histidine kinase and a response regulator (45). The M. tuberculosis genome encodes 11 complete two-component systems (11, 19), several of which contribute to the virulence of M. tuberculosis (33, 34, 36, 38, 49), but only one system (MtrA, MtrB) was found to be essential for cell growth (48). The relatively low number of two-component systems is offset by alternative signal transduction mechanisms involving Ser/Thr phosphorylation (2) that are generally less common in bacteria than in eukaryotes (26). M. tuberculosis has genes for one phospho-Ser/Thr phosphatase (pstP) and as many as 11 STPKs (pknA to pknl) (11). In mycobacteria with larger genomes, such as Mycobacterium marinum or Mycobacterium smegmatis, STPKs outnumber two-component systems, suggesting that the bulk of signal transduction is via Ser/Thr (de)phosphorylation.

Paradoxically, most of these STPKs do not appear to control essential physiological processes. Mycobacterium leprae, a closely related species that has undergone extensive gene decay (12), has retained only four STPKs, and orthologs of just three of them (pknA, pknB, and pknG) were found to be required for optimal growth of M. tuberculosis using saturation transposon mutagenesis (39). Furthermore, inactivation of the pknG gene in M. tuberculosis was reported to decrease viability both in vitro and upon infection of BALB/c mice (13), although independent work showed that the in vitro growth of Mycobacterium bovis BCG lacking pknG was identical to that of the wild type (29). In a similar way, wild-type-like growth was observed for M. tuberculosis strains lacking either the pknD or the pknH gene (32, 37), and downregulation of PknF protein synthesis in M. tuberculosis using an antisense strategy also confirmed a viable phenotype, with faster-growing and shorter cells than the wild-type strain (15).

In this work, we focus on the pknB gene, which is part of an operon that is strictly conserved in all known mycobacterial genomes and some related actinomycetes. Genes coding for a PknB-like protein kinase are also found in a large number of gram-positive bacteria (9). PknB is a receptor-like transmembrane protein, with an extracellular signal sensor domain and an intracellular kinase domain (4), that shares striking similarity in protein fold, catalytic machinery, and kinase regulation mechanism with eukaryotic STPKs (9, 31, 47). Here, we report the inactivation of the pknB gene in M. tuberculosis H37Rv and M. smegmatis mc²155 and provide the first direct evidence of the essentiality of a STPK gene in mycobacteria. We also present the effects of several known ATP-competitive inhibitors on PknB phosphorylation activity in vitro and demonstrate that strong PknB inhibitors can prevent the growth of slow-
and fast-growing mycobacterial species, highlighting the potential of STPKs as therapeutic targets for the development of new antituberculosis drugs.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Escherichia coli XL1-blue, used for cloning experiments, was routinely propagated in LB broth (Difco) at 37°C. M. smegmatis mc2155 (41) was grown at 30, 37, or 42°C in LB broth supplemented with 0.05%Tween 80 or in 7H9 broth (Difco) supplemented with 0.05% Tween 80 and albumin-dextrose-catalase (ADC). M. tuberculosis H37Rv was grown in 7H9 broth supplemented with 0.05% Tween 80 and ADC or on 7H11 agar medium supplemented with oleic acid-albumin-dextrose-catalase (ODC) at 30, 37, or 39°C. Antibiotics were added at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 20 μg/ml; hygromycin, 50 μg/ml. When required, 10% (M. smegmatis) or 2% (M. tuberculosis) sucrose was added to the solid medium.

Cloning procedures and Southern blot analysis. (i) Construction of pRBextinpknB and pOMK-pknB. Preparation of E. coli and mycobacterial electroporative competent cells, extraction of chromosomal DNA, Southern blotting, and cloning were carried out as described previously (24, 35). M. smegmatis genomic sequences were retrieved from TIGR (http://www.tigr.org) and used as templates. M. tuberculosis pknB and flanking regions were PCR amplified and cloned into the SpeI restriction site of the replicative plasmid pOMK (22) to obtain pOMK-pknB or were amplified using primers harboring SpeI and HpaI restriction sites and cloned into pRBextin (a kind gift from R. Brosch), an integrative cosmid vector derived from pYUB412 (5), to generate pRBextinpknB. All constructs were verified by sequencing. Details of the primers are available upon request.

(ii) Construction of the M. tuberculosis and M. smegmatis pkntn mutant. The essentiality of the pknB gene in M. tuberculosis and M. smegmatis was investigated following a standard strategy based on the construction of merodiploid strains (23). The ts-sacB method was used to achieve allelic replacement at the pknB locus of M. tuberculosis (23, 35), and pRBextinpknB was integrated to perform complementation experiments. A unique HindIII restriction site was created in the M. tuberculosis pknB gene by using the QiaClone site-directed mutagenesis kit (Stratagene) into which the kanamycin resistance cassette from pUC4K (Amersham Biosciences) was inserted. The resulting pknB-Km gene was then cloned into pPR27-xyEl-colored marker (35) to obtain pPR27pknB, the construct used for allelic replacement.

The essentiality of the M. smegmatis pknB gene was investigated using a two-step homologous recombination procedure to achieve allelic replacement at the pknB locus (23) and pRBextinpknB to perform complementation experiments. The M. smegmatis pknB gene and flanking regions were PCR amplified, and a disrupted allele, pknB-Km, was then obtained as described above. The construct used for allelic replacement, pQpknB, was obtained by cloning pknB-Km into pQ2000-xyEl (23).

Protein kinase assays. PknB and GarA were expressed in E. coli and purified as described previously (9, 43). Kinase assays were carried out in 15 μl of kinase buffer (50 mM HEPES [pH 7.1], 1 mM dithiothreitol, 0.01% Brij35, 5% glycerol, 2 mM MgCl2). All reactions were started with the addition of ATP and conducted at 30°C for 20 min. Myelin basic protein (MBP) phosphorylation assays were carried out with an enzyme:substrate molar ratio of 1:20 in the presence of 2.25 μCi of [γ-32P]ATP. The reactions were stopped by adding EDTA (15 mM, final concentration), and the proteins were resolved by adding 0.5 ml of kinase buffer containing 1 μCi of [γ-32P]ATP. The reactions were stopped by adding EDTA (15 mM, final concentration), and the proteins were resolved by SDS-PAGE and autoradiography, and the incorporation of radiolabeled ATP was quantified using a PhosphorImager (Synergy Software).

RESULTS

Genomics and experimental rationale. An inspection of the available genome sequences of mycobacteria, corynebacteria, and streptomycetes (1, 8, 11, 12, 20) revealed a conserved operon, near the chromosomal origin of replication, comprising five or six genes that may be involved in signal transduction pathways and cell division. In M. tuberculosis, this operon, in which the terminus codon of each gene overlaps the initiation codon of its follower, begins with Rv0019, coding for forkhead-associated (FHA) protein B. This is followed by an in-frame mycobacterial intergenic repetitive unit (42) which precedes pstP, encoding phosphoserine/threonine protein phosphatase (9), and the rodA and pbpA genes before the operon ends with the STPK genes, pkna and pknB (Fig. 1). Except for the presence of the mycobacterial intergenic repetitive unit, which is confined to M. tuberculosis, the same gene arrangement occurs in all sequenced actinobacteria, and the operon is transcribed counter to the direction of replication. In Streptomyces coelicolor, there is one less STPK gene, as pknA appears to have been lost (7). It has been speculated that these STPK genes may be essential and control cell division (3), although there is no formal evidence. To substantiate this claim, we have undertaken genetic and biochemical analyses of pknB which, as the distal gene in the operon, should be readily inactivated, without polar effects, unless it is essential.

Inactivation of pknB in M. tuberculosis. Attempts were made by allelic replacement to disrupt pknB in the pathogenic species, M. tuberculosis H37Rv, using the ts-sacB methodology (23, 35). However, despite our having performed three independent experiments, PCR analysis of clones corresponding to putative double crossovers recombinants showed that none of them exhibited the expected pattern for allelic exchange mutants. To investigate whether the failure to disrupt the pknB gene could be due to its essentiality, we next performed an allelic replacement experiment using a M. tuberculosis merodiploid strain. This strain was constructed by integrating a functional copy of pknB into the chromosome of M. tuberculosis H37Rv using the integrative cosmid vector pRBextinpknB. The integration of this cosmid at the tRNA 3′ end site of the chromosome was confirmed by PCR and Southern blotting. Gene replacement experiments were carried out as described above. Analysis by PCR and Southern blotting of clones corresponding to putative double crossover recombinants showed that, in most cases, the allelic exchange occurred at the inte-
rescue a M. tuberculosis pknB wild-type pknB mc2pJQpknB.5, were grown in LB-Km broth and then plated on LB-Km-hygromycin B-sucrose plates at 30°C as described above. The phenotype of approximately 1,000 Kmr-Hygr-Sucr colonies was tested for both types of transformants plated. No allelic exchange mutant (Km’-Hyg’-Suc’-XylE’ colony) was found when mc^pJOpkB.2/pRBexint or mc^pJOpkB.5/pRBexint was plated, confirming our previous results. In contrast, when mc^pJOpkB.2/pRBexint-pknB and mc^pJOpkB.5/pRBexint-pknB were plated, 30% of Km’-Hyg’-Suc’ colonies were found to be XylE negative. A Southern blot analysis of a recombinant clone revealed that it had undergone gene replacement at the pknB locus (Fig. 2b), indicating that pknB is also an essential gene in M. smegmatis.

Inactivation of pknB in M. smegmatis. To investigate whether the pknB gene is also required for M. smegmatis growth, a kanamycin-disrupted copy of the pknB gene, pknB::Km, was inserted into the sacB suicide vector pJQ200-XylE and introduced into wild-type M. smegmatis mc^c155 by electroporation. Kanamycin-resistant mc^pJOpkB.2 and mc^pJOpkB.5, were grown in LB-Km broth and then plated onto LB-Km-sucrose plates to select for clones that had undergone a second intrachromosomal crossover. Allelic exchange mutants are expected to carry the disrupted allele pknB::Km and to have lost the sacB and xylE genes carried by pJOpkB. However, the spraying of thousands of kanamycin-resistant sucrose-resistant colonies with catechol (to test for XylE’ strains) revealed that none of them exhibited the expected phenotype. Instead, these clones had probably undergone mutations in the sacB gene that conferred sucrose resistance.

To investigate whether the failure to disrupt pknB was due to its essentiality, we transformed the single crossover strains mc^pJOpkB.2 and mc^pJOpkB.5 with either pRBexint or pRBexint-pknB. Allelic exchange mutants were selected on LB-Km-hygromycin B-sucrose plates at 30°C as described above. The phenotype of approximately 1,000 Km’-Hyg’-Suc’ colonies was tested for both types of transformants plated. No allelic exchange mutant (Km’-Hyg’-Suc’-XylE’ colony) was found when mc^pJOpkB.2/pRBexint or mc^pJOpkB.5/pRBexint was plated, confirming our previous results. In contrast, when mc^pJOpkB.2/pRBexint-pknB and mc^pJOpkB.5/pRBexint-pknB were plated, 30% of Km’-Hyg’-Suc’ colonies were found to be XylE negative. A Southern blot analysis of a recombinant clone revealed that it had undergone gene replacement at the pknB locus (Fig. 2b), indicating that pknB is also an essential gene in M. smegmatis.

PknB inhibitors prevent mycobacterial growth. We next looked for strong PknB inhibitors and tested their effect on mycobacterial growth. Since the physiological substrate of PknB is unknown, the initial kinase assays to identify PknB inhibitors (within a panel of 18 commercially available compounds known to inactivate different eukaryotic STPKs) used MBP as a surrogate substrate (4, 9). As shown in Fig. 3a, significant inhibitory effects were observed, in particular for K-252-a and K-252-b, two natural products that contain the indole carbazole chromophore and are thought to target the ATP-binding site. It should be noted that, although the PKA inhibitor H-7 has been previously reported to inhibit PknB (16), we observed no effect of this compound on PknB, whereas it was able to inactivate mouse PKA at a 5 μM concentration in the present assay (data not shown).

Although PknB phosphorylates MBP in at least five different sites (17), MBP is a poor PknB substrate; a high enzyme:substrate molar ratio (1:20), which is probably unrepresentative of the physiological situation, has to be used in the kinase assay. We therefore used a proteomic approach to identify putative physiological substrates of PknB and identified the protein GarA, an FHA domain-containing protein, as the optimal PknB substrate in a soluble protein extract from M. tuberculosis (43). Based on the apparent kinetic constants, a new kinase assay was established using a PknB:GarA molar ratio of 1:2,000 and an ATP concentration of 2.25 μM, the...
apparent Km value for ATP. A screening of the panel of inhibitors using this assay confirmed the previous results, showing K-252-a, K-252-b, and staurosporine to be the strongest inhibitors (Fig. 3b). The IC50 values for these compounds were determined by quantification and graphical analysis of radiolabeled spots from serial twofold dilutions (Fig. 3c) and found to be 96 ± 7 nM for K-252-a (Fig. 3d), 106 ± 6 nM for K-252-b, and 0.6 ± 0.05 µM for staurosporine.

Using a colorimetric, resazurin microtiter assay (28), the antibacterial activity of compounds displaying a strong inhibitory effect in vitro was then assayed against *M. tuberculosis* H37Rv, *M. smegmatis* mc²155, and *Mycobacterium aurum* A+. The latter species is generally very sensitive to antitubercular drugs. The results of the susceptibility tests (Table 1) demonstrate that K-252-a inhibited the growth of both *M. tuberculosis* H37Rv and *M. smegmatis* mc²155 at a concentration of 20 µM and that of *M. aurum* A+ at 5 µM. As a control, staurosporine also showed inhibitory effects on *M. tuberculosis* H37Rv (25 µM < MIC < 50 µM). In contrast, K-252-b failed to inhibit the growth of all mycobacterial species at the highest concentration tested (40 µM), perhaps due to the low permeability of the envelope to this compound.

To further assess the correlation between PknB inactivation and the inhibition of mycobacterial growth, the *M. tuberculosis pknB* gene and flanking regions were cloned into pOMK, a mycobacterial replicative vector. The resulting plasmid, pOMK-pknB, was introduced into *M. smegmatis* mc²155, and the overexpression of PknB was assessed by Western blotting (data not shown). As a control, *M. smegmatis* mc²155 was also transformed with the empty pOMK vector, and the K-252-a resistance of the mycobacterial transformants was then assayed as described above. Two independent experiments showed that the MICs of the *M. smegmatis* mc²155 strains transformed with pOMK-pknB were twofold that of the control strain (Table 1), further suggesting that PknB is the actual molecular target in vivo.
Among the signaling elements in *M. tuberculosis*, only the MtrA response regulator has been previously found to be essential for growth (48). Although rare, other essential genes encoding two-component systems have been identified for gram-positive bacteria, in particular orthologs of *Bacillus subtilis* YycG-YycF (18) that are likely involved in cell division (21). However, despite the widespread occurrence of STPK genes in bacteria (2), no enzyme of this family has been demonstrated to be essential for bacterial viability. Our study now provides the first direct evidence that a eukaryotic-like Ser/Thr protein kinase, PknB, is essential for growth of both the pathogen *M. tuberculosis* and the saprophyte *M. smegmatis*.

What is the essential process(es) regulated by PknB? This kinase was first implicated as a potential regulator of cell growth and division, because of its localization close to the

**FIG. 3.** PknB inhibition assays. (a) Autoradiography of a sodium dodecyl sulfate-polyacrylamide electrophoresis gel showing PknB phosphorylation of MBP and the effect of different inhibitors at 100 μM. (b) Autoradiography of labeled spots on phosphocellulose paper showing the effect of the same inhibitors at 10 μM using a more sensitive phosphorylation assay (PknB phosphorylation of GarA). (a and b) Tested inhibitors were: lane 1, A-3; 2, calphostin C; 3, GF109203X; 4, H-7; 5, H-8; 6, H-9; 7, H-89; 8, HA-1004; 9, HA-1077; 10, hypericin; 11, K-252-a; 12, K-252-b; 13, KN-62; 14, KT-5720; 15, KT-5823; 16, ML-7; 17, ML-9; and 18, staurosporine. Positive (+) and negative (−) controls are shown. (c) Autoradiography of labeled spots on phosphocellulose paper showing GarA phosphorylation after serial twofold dilutions, starting at left from 10 μM (K-252-a and K-252-b) or 20 μM (staurosporine). (d) Dose-response plot of PknB fractional activity from five independent experiments as a function of K-252-a concentration. Similar plots were obtained for K-252-b and staurosporine. Vi and Vo, velocities in the presence and absence of inhibitors, respectively.
chromosomal origin of replication and since the operon also includes genes known to be important for these processes (rodA and pphA) (3). More recent results demonstrated that PknB is expressed predominantly during exponential growth (25) and upon infection of THP-1 human macrophages (40). Furthermore, the deletion or overexpression of PknB (and PknA) alters cell morphology, lending further support to its involvement in cell shape and cell division control (25). However, the physiological substrate(s) of PknB are currently unknown. The optimal PknB substrate in soluble protein extracts from M. tuberculosis was identified as GarA (43), an FHA domain-containing protein that has been linked both to glycosylation and to regulation of the tricarboxylic acid cycle in Mycobacterium smegmatis (6) and to regulation of the peptidoglycan (46) but, to our knowledge, no experimental evidence is currently available to support this claim.

PknB is conserved not only in actinobacteria (7)—a PknB-like protein kinase also is found in a large number of more distantly related gram-positive bacteria (9). In B. subtilis, the prkC (pknB-like) gene has also been proposed to control developmental processes, since disruption of the gene impairs sporulation efficiency and reduces biofilm formation (27). However, the mutant lacking prkC showed no differences in growth and morphology when compared with the wild-type strain, in contrast with our present results for mycobacteria. These observations suggest that pknB-like genes could have an ancient evolutionary origin in gram-positive bacteria, so that the biological processes that they control have significantly diverged in response to specific bacterial adaptation to the environment. Alternatively, PknB and PrkC may befunctionally unrelated, as prkC is neither located in an operon resembling that found in actinobacteria nor situated close to oriC.

To further assess PknB essentiality, we tested the effect of ATP-competitive STPK inhibitors on bacterial growth. Two out of 18 compounds tested (K-252-a and K-252-b) were found to inhibit PknB in vitro with an IC50 in the 100 nM range. The MIC of K-252-a against different slow- and fast-growing mycobacterial strains was found to be 5 to 20 μM. To address the question of whether PknB was the actual molecular target, we transformed M. smegmatis mc²155 with a multicopy replicative plasmid expressing M. tuberculosis PknB. This overexpressor exhibited a twofold increase in resistance to K-252-a compared with the control transfectant, supporting the hypothesis that PknB inactivation is related to the inhibition of bacterial growth.

Eukaryotic protein kinases have become one of the most important groups of drug targets (10), and large chemical libraries of specific protein kinase inhibitors are currently available. Thus, the evidence that PknB, a structurally and mechanistically eukaryotic-like STPK (31, 47), is an essential enzyme in M. tuberculosis and that PknB inhibitors, despite being selected from a small test panel, may have a significant antibacterial effect strongly suggests that STPKs may represent new therapeutic targets for antituberculosis drug design. We anticipate that further inhibitor screenings followed by compound optimization may lead to novel antibiotics of value against tuberculosis and note that other STPKs, such as PknG (44), may also be tractable targets.

ACKNOWLEDGMENTS

We thank Brigitte Boitel and Miguel Ortiz-Lombardia for their early contributions to this work and Annemarie Wehenkel, Marco Bellinzoni, and Roland Brosch for advice and materials.

This work was supported by grants from the Institut Pasteur (GPH-Tuberculosis) and the European Commission, contract nos. QLK2-CT-2001-02018 (X-TB) and LSHP-CT-2005-018923 (NM4TB).

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