

Essential Ser/Thr protein kinases as potential targets for the development of novel antibiotics against mycobacterial diseases

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Microbial genomics has confirmed the widespread presence of eukaryotic-like Ser/Thr protein kinases (and phosphatases) in prokaryotes. Indeed, the number of genes coding for these enzymes in mycobacterial genomes is similar to, or greater than, those coding for classical two-component systems, emphasizing an important role of reversible Ser/Thr phosphorylation in bacterial physiology and virulence. This presentation will review recent work on the essential Ser/Thr protein kinase PknB from *Mycobacterium tuberculosis*, establishing the proof of concept that protein kinase inhibitors are promising candidates for the development of novel anti-bacterial agents.

Tuberculosis (TB) is a major public health problem with one-third of the world's population infected by its etiologic agent, *Mycobacterium tuberculosis*, and over two million people dying from the disease each year. The Global Alliance for TB Drug Development has proposed to improve the current treatment by developing more potent therapeutic agents, which could act on latent and persistent bacilli and reduce the duration of therapy. To achieve these goals, it is imperative to discover new, unconventional drug targets.

M. tuberculosis has a complex life cycle. After inhalation, infectious bacilli are phagocytosed by alveolar macrophages in the lung and induce a local pro-inflammatory response, which leads to the recruitment of monocytes from the bloodstream into the site of infection^{1; 2}. By blocking fusion of phagosomes with lysosomes in these non-activated macrophages^{3; 4}, *M. tuberculosis* escapes killing and multiplies. As the immune response progresses, macrophages and T cells accumulate to form a granuloma in which the pathogen is contained in a latent state^{5; 6}. It can lie dormant for years only to rise again when the immune system wanes through old age, malnutrition or AIDS (Acquired Immuno-Deficiency Syndrome). The centre of the granuloma then liquefies and *M. tuberculosis* replicates profusely and is discharged into the bronchial tree producing an infectious cough¹. Regulatory proteins involved in mycobacterial signal transduction must therefore play a major role in adapting the bacterial response to these changes in host environment.

Signal transduction in prokaryotes is primarily conducted by two-component regulatory systems, basically consisting of a sensor histidine kinase and a response regulator⁷. The *M. tuberculosis* genome encodes 11 complete two-component systems^{8; 9}, several of which contribute to the virulence of *M. tuberculosis*¹⁰⁻¹⁴, but interestingly only one system (MtrA, MtrB) was found to be essential for cell growth¹⁵. This relatively low number of two-component systems is offset by alternative signal transduction mechanisms involving Ser/Thr phosphorylation⁷ that are generally less common in bacteria than in eukaryotes. *M. tuberculosis* has genes for one phospho-Ser/Thr phosphatase (pstP) and as many as 11 Ser/Thr protein kinases (STPKs) (pknA to pknL)⁸. In mycobacteria with larger genomes such as *M. marinum* or *M. smegmatis* STPK 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. Wild type-like growth was observed for *M. tuberculosis* strains lacking either the *pknD* or the *pknH* genes^{16; 17}, and downregulation of PknF protein synthesis in *M. tuberculosis* using an anti-sense strategy also confirmed a viable phenotype¹⁸. Indeed, *M. leprae*, a closely related species that has undergone extensive gene decay¹⁹, 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 mutagenesis²⁰. Furthermore, inactivation of the *pknG* gene in *M. tuberculosis* was reported to decrease viability²¹, but independent work showed that the *in vitro* growth of *M. bovis* BCG lacking *pknG* was identical to that of the wild type²². The most promising STPK target candidates, *pknA* and *pknB*, are part of an operon near the chromosomal origin of replication, which is conserved in mycobacteria, corynebacteria and streptomycetes^{8; 19; 23-25}. In *M. tuberculosis*, the operon comprises six genes involved in signal transduction pathways and cell division²⁶, including also *pstP* (encoding a phosphoserine/threonine protein phosphatase²⁷) and two other genes encoding FHA-domain proteins. We have focused our studies on the conserved Ser/Thr protein kinase PknB and its cognate phosphatase PstP.

The crystal structures of the catalytic domains of PknB and PstP have been determined by X-ray crystallography, confirming the extraordinary conservation of the protein fold and catalytic machinery between eukaryotic and mycobacterial homologs²⁸⁻³⁰. In two independent structures of PknB in complex with nucleotide triphosphate analogs, the activation loop was autophosphorylated but disordered, suggesting an induced fit mode of binding for the protein substrate(s). PknB crystallizes as a 'back-to-back' homodimer identical to that observed for the RNA-dependent protein kinase PKR³¹, suggesting a similar mechanism for kinase activation³². PstP was found to specifically dephosphorylate model phospho-Ser/Thr substrates in a Mn²⁺-dependent manner²⁷. Autophosphorylated PknB was shown to be a substrate for PstP and its kinase activity was affected by PstP-mediated dephosphorylation. Two threonine residues in the PknB activation loop, Thr171 and Thr173, were identified as the target for PknB autophosphorylation and PstP dephosphorylation. Replacement of these residues by alanine significantly decreased the kinase activity, confirming their direct regulatory role. These results indicate that, as for eukaryotic homologues, phosphorylation of the activation loop provides a regulation mechanism of mycobacterial kinases²⁷. A similar regulation mechanism could be also operational in other mycobacterial kinases, since mass spectrometry studies of four receptor-like protein kinases (PknB, PknD, PknE, and PknF) showed two recurrent clusters of autophosphorylation sites, respectively in the activation loops and juxtamembrane regions, all of which were dephosphorylated by PstP³³.

PknB was proposed to be a potential regulator of cell growth and division³⁴. Recent results demonstrated that PknB is predominantly expressed during exponential growth²⁶ and that its depletion or overexpression alters cell morphology²⁶, lending support to its involvement in cell shape and cell division control. However, the physiological substrate(s) of PknB are currently unknown. We identified the protein GarA as the optimal PknB substrate in soluble protein extracts from *M. tuberculosis* and the saprophyte *M. smegmatis*³⁵. GarA is a FHA domain-containing protein that has been linked both to glycogen degradation during exponential growth of *M. smegmatis*³⁶ as well as to regulation of the TCA cycle in *Corynebacterium glutamicum*³⁷. Enzymological and binding studies of the recombinant proteins demonstrate that docking interactions between the activation loop of PknB and the FHA domain of GarA are required to

enable efficient phosphorylation at a single residue, Thr22, of the substrate. The *garA* gene, including both the N-terminal phosphorylation motif and the FHA domain, is strongly conserved in mycobacteria and other related actinomycetes, suggesting a functional role of GarA in putative STPK-mediated signal transduction pathways³⁵. However, other putative PknB substrates have also been proposed, such as the penicillin-binding protein PbpA³⁸ or Rv1422²⁶.

Gene essentiality is a prerequisite for a target protein in anti-bacterial drug design. We demonstrated 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 gene³⁹. We also showed that eukaryotic Ser/Thr protein kinase inhibitors, which inactivate PknB *in vitro* with an IC₅₀ in the sub-micromolar range, have anti-bacterial activities against *M. tuberculosis* H37Rv, *M. smegmatis* mc²155, and *M. aurum* A+ with minimal inhibitory concentrations (MIC) in the micromolar range^{32; 39}. These findings demonstrate that the Ser/Thr protein kinase PknB is essential to sustain mycobacterial growth and support the development of protein kinase inhibitors as new, potential antituberculosis drugs.

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