

Protein phosphorylation is one of the principal mechanisms of signal transduction governing different cellular activities in both prokaryotes and eukaryotes. The enzymes catalyzing this process are known as protein kinases. They are broadly grouped into two superfamilies, histidine (His) and serine/threonine (Ser/Thr) or tyrosine (Tyr) kinases based on their sequence similarity and enzymatic specificity. In prokaryotes, phosphorylation events are known to be governed by His kinases, which autophosphorylate on histidine residue. On the other hand, such signalling in eukaryotes is mediated through cascades of protein phosphorylation on serine/threonine or tyrosine residues catalyzed by Ser/Thr or Tyr kinases. However, this compartmentalization of protein kinases has been offset in recent years by the discovery of His kinases in eukaryotes and of Ser/Thr or Tyr kinases in prokaryotes. The identification of *pkn1* gene from *Myxococcus xanthus* in 1991 marked the inception of eukaryotic-type Ser/Thr kinases in bacteria. Thereafter, in the past twelve years, the advent of genomics has revealed the existence of genes encoding Ser/Thr or Tyr kinases in a number of bacterial species. These bacterial homologues of eukaryotic-type kinases have largely been shown to be associated with growth and development as in *M. xanthus*, *Anabaena*, *Streptomyces* and *Bacillus subtilis*. Besides these, in certain bacteria, eukaryotic-type kinases have also been implicated in virulence/pathogenicity.

The genome sequence of *Mycobacterium tuberculosis*, the causative agent of the dreadful disease, tuberculosis, has unveiled the presence of a family of eleven eukaryotic-type Ser/Thr kinases. The existence of such a large number of regulatory proteins in this intracellular facultative pathogen could be attributed to its unique characteristics of being able to survive within the hostile environment inside the host together with its long quiescent dormant state. Therefore, the work embodied in this thesis is focussed on one such homologue of mycobacterial Ser/Thr kinases, PknA.

pknA was isolated from the genomic DNA of *M. tuberculosis* strain H37Ra using primers designed based on the genome sequence of the pathogenic strain, H37Rv. PknA was expressed in *Escherichia coli* in fusion with maltose binding protein (MBP) by cloning it in pMAL-c2. ~50% of the fusion protein was observed in the post-sonication supernatant. Subsequent affinity purification of the soluble

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fraction on amylose resin yielded a protein of molecular mass of ~97 kDa, which was recognized by anti-MBP antibody. However, the observed molecular mass of the purified fusion protein (MBP-PknA) was higher than that calculated from the nucleotide derived amino acid sequence (88.7 kDa). This did not seem to be unusual since a number of bacterial eukaryotic-type Ser/Thr kinases have been reported to show anomalous mobility on SDS-PAGE. The efforts to remove MBP tag from the fusion protein were unsuccessful due to non-specific cleavage of the protein by Factor Xa. Therefore, MBP-PknA was used in subsequent studies.

Ser/Thr kinases in both prokaryotes and eukaryotes have largely been shown to exhibit phosphorylating ability. *In vitro* kinase assays showed that the autophosphorylating ability of PknA was strictly Mg^{2+} / Mn^{2+} dependent. However, PknA autophosphorylation was stimulated significantly in the presence of Mn^{2+} compared to Mg^{2+} . Ca^{2+} , on the other hand, did not influence the enzymatic activity. Oxyanions (molybdate, tungstate and vanadate) are known to inhibit the phosphoryl transfer reactions. Therefore, the effect of different oxyanions when monitored, molybdate was found to be most effective in inhibiting the autophosphorylation of PknA. Phosphoamino acid analysis indicated that PknA was phosphorylated at serine and threonine residues. PknA was also able to phosphorylate exogenous substrates, like casein, histone and myelin basic protein. Furthermore, protein-protein interaction studies revealed the ability of PknA to phosphorylate at least a ~56 kDa soluble protein from *E. coli*.

Ser/Thr or Tyr kinases are annotated based on the presence of a catalytic domain harbouring eleven signature motifs, termed as Hank's subdomains. Analysis of the nucleotide derived amino acid sequence of PknA indicated the presence of these conserved subdomains towards its amino terminus end. PknA showed the ATP binding motif GXGXXG (alanine is present instead of first glycine and X is a variable amino acid) in subdomain I and an invariant lysine residue in subdomain II, characteristic of Ser/Thr or Tyr kinases. The essentiality of this lysine for exhibiting the kinase activity of PknA was highlighted by the inability of K42N mutant (lysine at amino acid position 42 of PknA was mutated to asparagine) to autophosphorylate. Furthermore, typical DXKPXN and GTXXYXAPE sequences of Hank's subdomain

VI and VIII respectively argued the inclusion of PknA as a member of eukaryotic-type Ser/Thr protein kinases. An Ala/Pro-rich (alanine/proline-rich) region was identified downstream to the catalytic domain of PknA. The deletion of this region despite the presence of the catalytic domain resulted in the loss of autophosphorylating ability. Ala/Pro-rich region was followed by a hydrophobic stretch of 23 amino acid residues. The charge distribution around this sequence of PknA revealed it to be a transmembrane kinase acting as a signalling molecule between the cell exterior and interior. The amino acid sequence following the transmembrane domain shared negligible homology with other eukaryotic-type Ser/Thr kinases and thus is unique to PknA. This stretch of amino acids presumably represents the regulatory domain of PknA.

In the *M. tuberculosis* genome, *pknA* (Rv0015c) is located adjacent to *pbpA* (Rv0016c) and *roda* (Rv0017c) genes, which encode putative morphogenic proteins belonging to SEDS (shape, elongation, division and sporulation) family. These SEDS family of proteins have been said to be present in all eubacteria where a constituent of cell envelope is the peptidoglycan. These proteins are known to be involved in the control of cell shape and peptidoglycan synthesis in bacteria like *E. coli* and *B. subtilis*. However, the presence of a Ser/Thr kinase in a cluster with morphogenic genes has so far not been reported in any bacteria. Thus, the location of *pknA* in *M. tuberculosis* genome is not only unique but also suggestive of its potential role in cell division/differentiation. Phylogenetic analysis of PknA with functionally characterized bacterial Ser/Thr kinases further indicated its possible involvement in these processes.

Alteration in cell shape is the initial event in bacterial cell division that involves ordered assembly of proteins. These proteins are fairly conserved among different prokaryotes. This is evident from the fact that a ~56 kDa soluble protein of *E. coli* interacted with mycobacterial PknA. In a preliminary study, cells of *E. coli* strain TB1 expressing MBP-PknA exhibited unusual elongation. To investigate the involvement of PknA in this process, the protein was expressed constitutively in the *E. coli* strain DH5 α using a *Mycobacterium* - *E. coli* shuttle vector, p19Kpro. As evaluated by scanning electron microscopy, expression of PknA resulted in dramatic

alteration in the phenotype of *E. coli* cells. The elongation of cells was about 20 to 30 fold compared to the normal rods of *E. coli*. This observation was not an experimental artifact or the effect of plasmid load since *E. coli* transformed with the vector (p19Kpro) as well as the antisense construct (p19Kpro-aPknA) did not show such phenotypic alteration. The elongation of cells did not seem to result in any toxicity from 'out of context' expression of the mycobacterial gene since growth curves between experimental and controls were very similar. Furthermore, the kinase dead mutant, K42N (cloned in vector p19Kpro), did not result in the elongation of *E. coli* cells, thereby indicating that the kinase activity of mycobacterial PknA is involved in regulating these morphological changes. Strikingly, Western blot analysis with cell lysates from *in vitro* culture of *M. tuberculosis* using antisera generated against MBP-PknA revealed the downregulation of PknA in stationary phase, which represents a state of limited metabolism with little or no cell turnover. Collectively, these lines of evidence convincingly established the participation of mycobacterial PknA in the process of cell division/differentiation. However, such morphological events need to be tightly regulated in order to maintain a state of homeostasis. Interestingly, a putative and the only Ser/Thr phosphatase, *ppp* (Rv0018c) in *M. tuberculosis* is encoded by the operon comprising of the Ser/Thr kinase, *pknA* and the morphogenic genes, *pbpA* and *rodA*. The phosphatase, *ppp*, has been considered to be genetically linked to this Ser/Thr kinase, suggesting the regulation of phosphorylation/dephosphorylation events. Since the present study established the auto- and substrate phosphorylating ability of PknA, it was tempting to envisage whether PPP affects the enzymatic activity of this kinase.

In order to initiate studies with PPP, the gene was PCR amplified from *M. tuberculosis* strain H37Ra and subsequently expressed as MBP fusion protein in *E. coli*. The phosphatase activity of PPP was monitored using p-nitrophenyl phosphate (pNPP) as the substrate. PPP was found to be a Mn^{2+} / Co^{2+} dependent protein phosphatase. The kinetic studies revealed a K_m of ~ 2 mM and a V_{max} of ~ 4 nmoles/min/mg protein. The phosphatase activity of PPP was inhibited by both EDTA and sodium fluoride. However, it was unaffected in response to either okadaic acid or sodium vanadate. These results corroborated well with the phylogenetic

analysis data that placed PPP as a member of PP2C family of protein phosphatases. Furthermore, the amino acid sequence analysis of PPP revealed the presence of eleven signature motifs containing eight invariant residues at its amino terminus end, typical of PP2C phosphatases. Mutations in two of these conserved amino acid residues, aspartate of motif II (D38A) and glycine of motif V (G117D) completely abolished the enzyme activity.

Inspection of the bacterial genomes has revealed the genetic linkage between Ser/Thr kinases and phosphatases, suggesting that phosphatases are reversing protein phosphorylation reactions catalyzed by linked kinases. Studies with PPP revealed its ability to remove phosphate from autophosphorylated PknA as well as the substrates phosphorylated by this kinase. These observations thereby established that genetically linked mycobacterial Ser/Thr kinase and phosphatase (PknA-PPP) form a functional unit *in vitro*.

Finally, our results revealed that *pknA* from *M. tuberculosis* encodes an active Ser/Thr kinase. An interesting finding of the present study is the remarkable elongation of *E. coli* cells observed upon the expression of PknA. Although, the involvement of this kinase in the process of cell division/differentiation in its natural host needs to be envisaged, nonetheless the evidence presented here strongly suggested the participation of *pknA* in regulating these morphological changes. Since bacterial Ser/Thr kinases and phosphatases have been reported to have opposing physiological roles, it remains to be seen how *ppp* behaves in this situation. However, the ability of PPP to interact *in vitro* with PknA is definitely a pertinent information towards elucidating the role of phosphorylation/dephosphorylation cascade in the process of mycobacterial cell division/differentiation.