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Homology modelling, docking, pharmacophore and site directed mutagenesis analysis to identify the critical amino acid residue of PknI from *Mycobacterium tuberculosis*

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ABSTRACT

Tuberculosis is caused by *Mycobacterium tuberculosis*, an intracellular pathogen. PknI is one of the 11 functional Serine/Threonine Protein Kinases which is predicted to regulate the cell division of *M. tuberculosis*. In order to find newer drugs and vaccine we need to understand the pathogenesis of the disease. We have used the bioinformatics approach to identify the functionally active residues of PknI and to confirm the same with wet lab experiments. In the current study, we have created homology model for PknI and have done comparative structural analysis of PknI with other kinases. Molecular docking studies were done with a library of kinase inhibitors and T95 was found as the potent inhibitor for PknI. Based on structure based pharmacophore analysis of kinase substrate complexes, Lys 41 along with Asp90, Val92 and Asp96 were identified as functionally important residues. Further, we used site directed mutagenesis technique to mutate Lys 41 to Met resulting in defective cell division of *Mycobacterium smegmatis* mc^2. Overall, the proposed model together with its binding features gained from pharmacophore docking studies helped in identifying ligand inhibitor specific to PknI which was confirmed by laboratory experiments.

1. Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) *is* one among the devastating disease that kills 1.4 million people every year [1]. In spite of global awareness for drug resistant TB, Multi drug resistant (MDR) and extensively drug resistant (XDR) TB continue to emerge in high HIV prevalence setting, and the mortality rate in HIV co-infected patients remains high. *Mycobacterium bovis* Bacillus Calmette Guerin (BCG), the currently available vaccine, can be used only for severe forms of childhood TB, but its efficacy against pulmonary TB in adults remains controversial [2]. The success of *M. tuberculosis* results from its remarkable capacity to survive within the infected host, where it can persist in a non-replicating state for several decades. Protein phosphorylation is the principal mechanism involved in translating extracellular signals into cellular responses and this process is carried out by specific protein kinases [3].

M. tuberculosis genome consists of 11 Serine Threonine Protein Kinases (STPKs) (PknA–PknL). Among the 11 STPKs, nine are transmembrane receptors with kinase domain located within the cell (PknA, PknB, PknD, PknE, PknF, PknH, PknI, PknJ and PknL) and two kinases are soluble proteins (PknG and PknK) [4,5]. These STPKs have been suggested to play significant roles by influencing diverse signalling pathways depends upon the bacterial environmental conditions. PknA, PknB and PknF was functionally characterized and found to play a role in cell division [6–10]. Besides cell division PknF also plays a role in biofilm formation and glucose transport [10,11]. Five of the STPKs namely PknI, PknE, PknG, PknH, and PknK are reported to support intracellular survival [12–16]. PknL plays a role in starvation response [17].

In analogue with other *M. tuberculosis* STPKs, PknI also possesses N-terminal cytosolic domain and C-terminal extracellular domain. The C-terminal domain of STPKs is thought to sense environmental changes, which are communicated to the internal milieu through the N-terminal domain constituting the active site of the kinase [18]. Based on the sequence analysis of the kinase domain of *M. tuberculosis* STPKs, PknI is positioned in the same clade as that of PknF and PknJ [19]. These observations provide some insights into

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the evolution of PknI. In the *M. tuberculosis* genome, PknI, is flanked by the DacB2, FtsY and Ffh genes involved in cell division and protein secretion, indicating a possible role for PknI in one of these processes [4]. The sequence comparison analysis of PknI with other prokaryotic STPKs revealed its close homology to Stk1 from *Streptococcus agalactiae*, which was shown to play a role in the virulence and cell segregation of the organism. The genes for PknA, PknB and PknI, are located within the same operons that are known to be involved in cell division in *Escherichia coli* and *Bacillus subtilis*; thus it has been suggested that PknI may be involved in the regulation of bacterial cell division [4]. Previously, we have reported that PknI has a functionally active kinase activity and showed manganese dependent auto-phosphorylation at serine and threonine residues [20].

We found that the, PknI over-expression, leads to growth retardation and cell elongation in non pathogenic *Mycobacterium smegmatis* mc^2 (unpublished data). The PknI knockout strain of *M. tuberculosis* regulates the growth under stress conditions like acidic pH and low oxygen availability. PknI plays a role in sensing the macrophages host environment and translating it to slow the growth of *M. tuberculosis* within the infected host. Moreover, PknI Knockout strain of *M. tuberculosis* showed hyper-virulence phenotype in severe combined immunodeficiency (SCID) mice when compared to wild type strain [12]. Overall, we demonstrated that the PknI has a role in cell division and virulence of *M. tuberculosis*.

PknI has two unique distinguishing features when compared with other kinases. Firstly, though the PknI has a transmembrane domain surprisingly it's localized predominantly in the cytosol. Secondly, the expression pattern of PknI differs from that of other kinases, as exemplified by comparison with PknA and PknB: PknI decreases significantly over the course of infection of macrophages, while expression of PknA and PknB increases [21].

For the current study, we have used computational approach to predict the three dimensional structure of PknI. Homology modelling approach produces valid structural models for protein sequences with available related templates (having > 30 percentage amino acid sequence identity) [22]. Comparative study of these models with the known protein structures in Protein Data Bank (PDB) will help in understanding the similarities that may also facilitate inferring biochemical and biological functions [23].

In the present study, we have developed three dimensional model for PknI protein in order to predict the evolutionarily conserved and functionally important amino acids which are crucial for its kinase activity. This was further proved by using site directed mutagenesis studies. A pharmacophore model was developed by using both structure and ligand based methods. The best model was used to screen a library of compounds using GOLD docking methodology.

2. Materials and methods

2.1. Sequence alignment, secondary structure prediction

Protein sequence of *M. tuberculosis* Ser/Thr protein kinase, PknI (Swissprot: P65730) was used. ClustalW [24] and Align Multiple Sequences of Discovery Studio (DS) v 2.0 enabled the alignment of multiple sequences. Jpred: A Consensus Secondary Structure Prediction Server was used to predict secondary structure elements of PknI. Insertions were manually adjusted to preserve secondary structural features in PknI.

2.2. Template selection and homology modelling

The amino acid sequence of PknI protein was subjected to PSI-BLAST [25] and Phyre2 [26] to identify the most suitable crystal structure in PDB [27] database as template for modelling PknI. The best template was selected based on sequence identity, domain coverage, resolution, E-value and bound with cognate ligand. Based on sequence search and fold recognition, 1O6Y, PknB kinase from *M. tuberculosis*, was selected as template (32% identity and 3e−25 *E*-value). We used the homology modelling procedure such as MODELLER [28] which works well in the range of 30–40% identity between sequences. MODELER is able to simultaneously incorporate structural data from one or more reference proteins. Structural features in the reference proteins are used to derive spatial restraints which, in turn, are used to generate model protein structures using conjugate gradient and simulated annealing optimization procedures. Homology modelling of PknI was done using MODELLER, Discovery Studio v 2.0. We used PROCHECK [29] to check the geometry and stereochemical quality of our predicted structures and environment profile using VERIFY-3D [30] graph. Models were developed using different alignments and the best model was selected for further analysis. The final model was also superimposed with the template structure using PBDefold [31] for calculating the backbone RMSD.

2.3. Docking of substrate and drugs

Gold (Genetic Optimization for Ligand Docking, version 5.2) was used to dock the substrate and drugs in the active site to obtain the conformation and orientation. GOLD uses a genetic algorithm methodology for protein ligand docking that allows full ligand and partial protein flexibility. The modelled PknI structure was used for docking process, hydrogen atoms were added. The ATP and drug molecules were corrected using the LigPrep and Auto Edit Ligand option in Schrodinger and GOLD program respectively. The binding site of ATP was mapped based on known kinases complexes present in PDB and was provided an input for GOLD calculation. Default genetic algorithm (GA) settings that ensure 100% search efficiency were used for docking. An early termination of the number of GA runs was allowed when the RMSDs of the top three GA solutions were within 1.5 Å. The best pose of the docked ligand was selected based on CHEMPLP score.

2.4. Site directed mutagenesis of PknI

The site directed mutagenesis was done as described previously [11]. Briefly, PCR amplifications were done using Taq DNA polymerase (Roche) and genomic DNA of *M. tuberculosis* H37Rv as the template. Restriction enzyme recognition sites were added to the primers to facilitate in frame and directional cloning as indicated. The PknI coding region was PCR amplified using the oligo 1 and 2 (Table 1), and cloned into the BamHI and EcoRV site of pSD26 containing an acetamidase promoter and C-terminal His tag to create pRG5 (Table 1). The *PknI* gene was cloned without its stop codon in frame to a His tag and a stop codon was introduced immediately after the tag. The coding region of *PknI* was amplified using the primers, oligo 3 and 4 as primers and cloned into the pMV261 which carried an *hsp60* promoter. Replacement of the ATP-binding domain lysine (K) at position 41 to methionine (M) was performed using a Quik-Change site directed mutagenesis kit (Stratagene) following the supplier's instructions. The primers used for creating lysine to methionine mutation were Oligo 5 and 6 (Table 1). The sequences of all the constructs were confirmed by automated sequencer (Applied Biosystem Genetic Analyzer, model 3100).

2.5. Overexpression of PknI gene in M. smegmatis mc^2

Plasmids pSD26, pRG5 and pRG5–K41M were introduced into *M. smegmatis* by electroporation. Mycobacterial cells were lysed