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CarD-a reliable target in *M.tuberculosis*

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ABSTRACT :Tuberculosis remains a major health problem being the second leading cause of death from an infectious disease worldwide. The largest increases between 2011 and 2012 were in India, South Africa and Ukraine. (WHO Global tuberculosis report - 2013). Because most TB drugs are less efficient in killing slowly replicating or dormant bacilli in the chronic phase of TB infection, a key challenge for identifying sterilizing drugs stands before humanity. Identifying potential targets in the organism *M.tuberculosis* will aide in rapid drug finding process. One recommended target is CarD. CarD proteins are highly conserved in many eubacteria and are absent from archaebacteria and eukaryotes (Cayuela et al., 2003), but its exact function is yet to be discovered. It is revealed to be an transcription factor protein in *M.tuberculosis* and has been found to be essential for the organisms survival in the stringent host environment. Of the two domains –the N-terminal domain interacts with RNA polymerase and is said to regulate transcription of few ribosomal proteins and rRNA. The C-terminal domain, whose function was not known for a long time is suspected to interact with DNA and has a unique fold. The predictions and analysis of this protein, regarding its importance in survival of the organism, its structure, along with its known and suspected function in various studies are reviewed in this paper.

***KEYWORDS*:** CarD protein, *M.tuberculosis, transcription regulation*

I. INTRODUCTION

During Tuberculosis infection, in the lungs, within the granuloma, the infected macrophages are activated to destroy the bacteria- *M. tuberculosis* with which they are infected (Kaufmann SH., 2002). The infected cells restrain mycobacteria from proliferating by imposing an arsenal of oxidative stress, hypoxia, acid stress, genotoxic stress, cell surface stress, and starvation. Despite this onslaught of attacks, *M. tuberculosis* is able to persist for the lifetime of the host, indicating that this pathogen has substantial molecular mechanisms for resisting host defenses. To persist in this hostile environment *M.tuberculosis* enters a state of dormancy and rapidly down regulates ribosome biogenesis to match declining translational need. Generally, Bacteria accomplishes this via the stringent response, a global regulatory mechanism in which transcription of stable RNAs is inhibited, in part by the production of the hyperphosphorylated guanine nucleotides ppGpp and pppGpp (Avarbock et al., 2000; Magnusson et al.,2005). DksA protein potentiates the effect of (p)ppGpp by directly binding the RNAP (Paul et al.,2004). DksA homologs are absent in some divisions of bacteria, whereas (p)ppGppsynthetases are broadly distributed. Recent work (Stallings et al., 2009) has identified CarD protein interacts with RNAP β subunit to control rRNA transcription in *Mycobacterium tuberculosis* and this interaction is vital for *Mtb*'s survival during the persistent infection state. It was found that CarD is essential for viability, in that declining carD transcript levels directly correlated with cell death. CarD depletion leads to an up regulation of 16s rRNA and rpsH ribosomal protein transcripts in the organism. Mutant cells lacking CarD were found to be highly susceptible to antibiotics, when compared to wild strains. Hence CarD is suggested to be a potential drug target (Stallings et al., 2009).

II. CarD

M. tuberculosis carD (Rv3583c) has earlier been identified as a transcriptionally upregulated gene in microarray experiments after exposure to the DNA-damaging agents mitomycin C, UV radiation, H2O2, and quinolone DNA gyrase inhibitors (Boshoff et al., 2004).Also earlier work in M. xanthus had identified a paralog of CarD that contains an HMGA domain and is said to be involved in fruiting body formation and carotenogenesis but which is nonessential (Padmanabhan,2001). The same research group examined the function of the M. xanthus ortholog of mycobacterial CarD called CdnL (CarD N-terminal Like).

(Garcia-Moreno,2010) to distinguish this protein from the HMGA domain containing CarD. Studies in *B.burgdorferi* have also identified a CarD homolog, LtpA, that is induced at low temperatures(Yang XF.,2008).

III. ESSENTIABILITY

A broad range of experiments and assays were carried out by Stallings et al, which threw light on the essentiability of this protein in *M.tuberculosis* and *M.smegmatis*. In one experiment, the transcriptional induction of carD after I-SceI-generated double stranded break in *M.smegmatis* was quantified by DNA microarray and verified by quantitative real-time PCR (qRT-PCR).Wild-type *M. smegmatis* cultures when treated with double-strand DNA-damaging agents bleomycin and Ciprofloxacin, alkylating agent methyl methanesulphonate (MMS), and the oxidizing agent hydrogen peroxide (H2O2), it was observed that carD transcript increased with all genotoxins tested except high doses of H202, with the most dramatic induction observed with Ciprofloxacin and 10 mM H2O2, which induced carD transcription 8-fold compared to untreated cells. Survival assays and quantitative RT-PCR showed that declining carD transcript levels directly correlated with cell death which indicated that CarD is essential for viability. In the other experiment, it was found that mycobacterial cells lacking CarD were 50,000-fold more sensitive to 10 mM H2O2 and 30 times more sensitive to Ciprofloxacin treatment compared to control cells producing CarD. also CarD depletion caused a 10-fold reduction in *M. smegmatis* survival during starvation. These findings indicated that CarD is necessary for survival during oxidative stress, double stranded DNA breaks and nutrient limitation. Also, with *M. smegmatis* cells, the microarray analysis revealed that during CarD depletion, 193 genes were upregulated >2-fold and 176 genes were downregulated >2-fold. Later, the microarray and qRT-PCR experiments in *M. smegmatis* and *M. tuberculosis* demonstrate that CarD depletion leads to transcriptional upregulation of stable RNAs and other components of the translation machinery such as 16S rRNA and rpsH ribosomal protein transcripts. Also in their studies it was found that *M. tuberculosis* CarD is necessary for replication and persistence during infection of mice (Stallings et al.,2009). Chromatin immunoprecipitation (ChIP) experiments in *M. smegmatis* with antibodies targeted against CarD precipitated the promoters regulating the rRNA operons or the rplN operon (encoding ribosomal proteins L14, L24, L5, and S14p/S29e), thus confirming that CarD is physically present on the RNAP complex at the rRNA and ribosomal protein loci. It was analyzed that the microorganisms in which CarD is known or suspected to be essential (*Mycobacteria*, *M. xanthus* and *B. burgdorferi*) all contain 4 or fewer rRNA operons (Stallings et al.,2009; Garcia-Moreno et al.,2010; Yang XF et al.,2008) Whereas, in organisms like *B. subtilis*, where CarD is non essential 10 or more rRNA operons are found. (Kobayashi et al 2003)

IV. COMPARISON WITH DKSA

It was known that the universal protein (p)ppGpp participates in stringent control by downregulating rRNA formation. In E. coli, the DksA protein potentiates the effect of (p)ppGpp by directly binding the RNAP. DksA protein decreases the open complex half- life during transcription initiation and thereby known to downregulate transcription from the P1 promoter of the *E.coli* rRNA rrnB operon. In a DksA deletion strain, rRNA transcription is insensitive to (p)ppGpp accumulation, thus leaving rRNA promoters unresponsive to changes in amino acid availability and growth rate (Paul et al., 2004). Deletion of DksA has pleiotropic effects including DNA damage sensitivity which have not been clearly linked to its effect on transcription initiation (Branny et al.,2001; Magnusson et al., 2007). Despite the intricate functional relationship between DksA and (p)ppGpp in E. coli, obvious DksA homologs are absent from most nonproteobacteria, whereas (p)ppGpp synthetases are broadly distributed, possibly indicating that other factors exist to control ribosomal biogenesis in these organisms.In mycobacteria, a protein CarD was found to bind the RNAP to control rRNA transcription.To test whether CarD could replace DksA in E. coli, *M.smegmatis* CarD is expressed in ΔdksA strain (Magnusson et al., 2007) from an E. coli promoter. This restored ΔdksA growth on minimal media almost to the extent as DksA itself. During nutrient limitation in the ΔdksA strain, rRNA levels was measured during starvation and found that CarD was able to decrease the elevated rRNA levels indicating that CarD could replace DksA in the stringent response.

Structural modeling and experimental evidence support a model in which DksA exerts its effects on the RNAP through interactions within the secondary channel. In contrast, the CarD N-terminus has a TRCF RID(Transcription repair coupling factor - RNA polymerase interacting Domain) module and interacts with the N terminus of the RNAP β subunit, where TRCF binds. Accordingly, TRCF RID alone can mimic the effect of CarD on rRNA transcription in an *E.coli* DdksA strain. These results suggest that CarD affects rRNA transcription through a different mechanism than DksA. Thus CarD can complement an *E. coli* DdksA strain, but in contrast to *E. coli* DksA, CarD is required for mycobacterial viability under all conditions, both in culture and during all stages of mouse infection whereas *E. coli* DksA is dispensable in nutrient-rich media (Stallings et al, 2009). Loss of DksA or CarD causes similar phenotypes despite having different interaction sites on RNAP. In a recent paper, Tehranchi et al. show that, in addition to its role in regulating rRNA transcription initiation,

DksA ensures progression of DNA replication in *E.coli* by removing transcription roadblocks during nutrient deprivation. This leads to a speculation that CarD might play a role in resolving conflicts between transcription and DNA replication and that this function is required for the viability of the mycobacterial cell (Stallings and Glickman,2010).

V. COMPARISON WITH TRCF

TRCF (Transcription repair coupling factor), encoded by the mfd gene, is necessary for DNA strand-specific repair during transcription. A lesion in the template strand blocks the RNA polymerase complex. The RNAP-DNA-RNA complex is specifically recognized by TRCF which releases RNAP and the truncated transcript. The TRCF may replace RNAP at the lesion site and then recruit the uvrA/B/C repair system (Westblade et al.,2010;Chambers et al.,2003). Abundant evidence indicates that the TRCF RID(RNAP β Interacting Domain) interacts directly with the N terminus of the RNAP βsubunit (Deaconescu et al.,2006). Earlier,with bacterial two-hybrid assay, it was noticed that the N-terminal domain of *T. thermophilus* CarD, which shares homology with the TRCF RID and M. tuberculosis CarD, interacts with the N-terminus of RNAP β (amino acids 10–133) to the same degree as did the TRCF RID. Full-length *T. thermophilus* CarD also interacted strongly with RNAP β amino acids. These data demonstrate that the mode of binding of CarD to RNAP is the same as TRCF. Despite the similarities between the N terminus of CarD and the TRCF RID, attempts to complement CarD depletion with the *M. smegmatis* TRCF RID were unsuccessful, indicating that RNAP binding is not sufficient for CarD activity. Additionally, an *M. smegmatis* Dmfd strain does not phenocopy depletion of CarD in that TRCF is not essential in mycobacteria, loss of TRCF does not affect mycobacterial sensitivity to oxidative or genotoxic stress, and rRNA levels are regulated normally in Dmfd both during normal growth and in conditions that elicit stringent control. Therefore, despite the similar mechanism of interaction with the RNAP, CarD and TRCF have distinct cellular functions (Stallings et al.,2009).

Comparison of the predicted 3D structure of CarD of *M.tuberculosis* with TRCF RNAP-β interacting domain (TRCF-RID; PDB ID: 3MLQ) and *Thermus thermophilus* CdnL-NTD [PDB ID: 2LQK) predicted that one of the major differences between the structures of CarD-NTD and TRCF-RID/CdnL-NTD lies in the curvature of β sheet formed by β1 and β2 strands. The reduced curvature in the β sheet of CarD-NTD may have a physiological implication, as one of the key interactions predicted between the CarD R25 residue and RNAP-β E1385 would require large conformational rearrangements. High B-factor in this region of the CarD structure, suggests the possibility for such conformational rearrangements (Kaur et al.,2013).The X-ray crystal structure (PDB ID 3MLQ) of the *T. thermophilus* TRCF RID complexed with the *Thermus aquaticus* RNAP β1 domain revealed that *T. thermophilus* TRCF RID R341 forms a hydrogen bond with RNAP β1 Q99, as well as polar interactions with RNAP β1 E110. The RNAP β1 residues I108 and K109 are also central to the protein/protein interface, making extensive van der Waals contacts with the TRCF RID .To extrapolate these structural insights to mycobacteria, the *T.aquaticus* β1 sequence was aligned with the β1 domain from *M.tuberculosis* and concluded that residues E138, I147, K148, and S149 of *M. tuberculosis* RNAP β1 correspond to residues Q99, I108, K109, and E110 in *T.aquaticus* RNAP β1, respectively. Bacterial two-hybrid experiments were performed between the *M. tuberculosis* TRCF RID and RNAP β1 and between *M.tuberculosis* CarD RID and RNAP β1 harboring one or more mutations in these residues.

The *M. tuberculosis* RNAP $\beta1^{E138R}$ and RNAP $\beta1^{I147A}$ substitutions had dramatic effects on the strength of the interaction with the TRCF RID and CarD RID by weakening the interaction. In contrast, alanine substitutions at *M. tuberculosis* RNAP β1 K148 or S149 did not inhibit the association with the TRCF RID but weakened CarD RID binding. They therefore concluded that, CarD and TRCF associate with the RNAP β subunit in similar manners, although with subtle differences (Weiss et al.,2014).Later when M.tuberculosis CarD/RNAP complex structure was solved, comparison of *Mtb* CarD/RNAP and *Tth* TRCF-RID/β1 complex structures reveals that CarD and TRCF-RID display a similar set of interactions with RNAP, even though there is no sequence conservation between the CarD β4- (43DLTVRVP49) and TRCF β4- (358EGKLYLP364) strands that interact with the RNAP β1 domain except for the last proline residues(Gulten et al.,2013).In a computational study, the proteins interacting with CarD and TRCF of *Mycobacterium* species was known by sequence comparison against STRING database and were compared and analyzed. DNA directed RNA polymerase subunit β and 50 s ribosomal protein L25 are common proteins who have functional association with CarD and TRCF and relates to their common function. Two proteins 2-C-methyl –D-erythritol 2,4 –cyclodiphosphate synthase and 2-c-methyl-D-erythritol 4-phosphate cytidylyl transferase, both of which are involved in isoprenoid biosynthesis pathway and are already recognized as drug targets were found to be functionally associated with CarD (based on its genomic neighborhood) and not with TRCF. As Isoprenoids are essential for the survival of the organism (Heuston et al,2012;Beutow et al,2007) this gives a clue of possible function of CarD other than RNAP interaction ,which makes it essential for the survival of the organism, when compared with TRCF(Priya et al.,2012).

VI. N-TERMINAL DOMAIN (NTD)

Earlier, being not able to obtain suitable crystals of *M.tuberculosis* CarD, Srivatsa et al, determined the structure of CarD from *Thermus thermophilus* at 2.4 Å resolution, as it has 44% sequence similarity with *M.tuberculosis* CarD. The *T. thermophilus* CarD structure, was found to comprise of Tudor-like fold of the N-terminal domain, in common with the *E.coli* TRCF-RID. *T.thermophilus* CarD was crystallized with two molecules in the asymmetric unit, so two crystallographically independent structures were refined. Despite having unique crystal packing environments, the two molecules are nearly identical in structure (rmsd of 0.965 Å over 158 α-carbon positions), indicating that the relative orientation of the two CarD structural domains is rigidly maintained. This is likely due to a small but significant interface between the two domains (~810 Å2 buried accessible surface area) that includes a network of conserved interactions. Two conserved charged residues (CarD-RID-R60 and CarD-CTD-E139) form a partially buried, inter domain salt bridge. CarD-RID-R60 is also in position to hydrogen bond with the carbonyl-oxygen of conserved CarD-CTD-G100. Conserved hydrophobic residues V11 and V17 make van derWaals interactions with the alkyl chains of R60 and E139 as well as with the side chain of CarD-CTD(C-terminal domain) residue 142 (Q in T. thermophilus CarD, but conserved as a hydrophobic residue in the larger family of CarD proteins) (Srivastava et al.,2013). Later with the crystal structure (PDB ID:4ILU) it was confirmed that CarD of *M.tuberculosis* has two domains, where the N-terminal domain (CarD-NTD) forms an antiparallel β sheet (β1–β3) and the C terminal domain (CarD-CTD) adopts predominantly an all-α (α1-α5) conformation, a characteristic of (α+β) family of proteins (Kaur et al.,2013).Also in the structure (PDB ID: 4KBM) *M.tuberculosis* CarD is seen to composed of two distinct domains: an all β-stranded N-terminal domain (residues 1–49) and an all α-helical C-terminal domain (residues 63–160). The N- and C-terminal domains are connected by a six residue twisted α helix (α1) and an eight residue loop. The N-terminal domain has a Tudor-like fold (Selenko et al., 2001) consisting of four anti parallel β strands. Residues Thr26, Ile27, Lys28, and Gly29, which lie on the β-turn connecting the β2 and β3 strands, were the only residues disordered in the N-terminal domain(Gulten et al.,2013).

Interaction with RNA polymerase:

To identify residues in CarD that are important for the CarD/RNAP interaction, a homology model of the *M. tuberculosis* CarD RID interacting with the RNAP β1 domain was generated based on the structure of the *Thermus* sp. TRCF RID/RNAP β1 complex (PDB ID 3MLQ). According to modeled structure, *M.tuberculosis* CarD RID R25 interacts with *M. tuberculosis* RNAP β1 E138 (homologous to the *Thermus* sp. TRCF-RID R341: RNAP β1 Q99 interaction). In addition to *M. tuberculosis* CarD RID R25, the model structure predicts that another arginine that at position 47, would interact with *M. tuberculosis* RNAP β1 E138. Arginine-to-glutamic acid substitutions at position 25 or 47 in CarD RID bacterial two-hybrid constructs was made and CarD and RNAP β1 interaction was tested. Either substitution on its own greatly diminished the ability of these proteins to interact, with CarD RID^{R25E} having the most severe effect. A double point mutation (CarD $RID^{R25E,R47E}$) completely abolished the interaction. They were able to fully suppress the effect of the CarD RID^{R47E} mutation with the RNAP $\beta1^{E138R}$ substitution, confirming the specific interaction between these two residues predicted from the structural model. The RNAP $\beta1^{E138R}$ mutation did not suppress the effects of the CarD RID^{R25E}mutation, suggesting that the CarD RID^{R25E} substitution disrupts additional contacts between these proteins. This is supported by the *Thermus* sp. TRCF RID/β1 structure, where *Thermus* sp. TRCF RID R341 (corresponding to *M. tuberculosis* CarD RID R25) interacts with multiple residues in the β1 subunit. The carD gene of *M. smegmatis* and *M. tuberculosis* was replaced with the alleles encoding $CarD^{R25E}$, $CarD^{R47E}$, or the $CarD^{R25E,R47E}$ double mutant using a gene-switching technique .It was not possible to engineer a viable $CarD^{R25E,R47E}$ double mutant, which is incapable of interacting with RNAP β1, indicating that the interaction between CarD and the RNAP is important for viability. In fact, even the $CarD^{R25E}$ substitution was unattainable in *M. tuberculosis*, again emphasizing that attenuating the CarD/RNAP β1 interaction compromises survival (Weiss et al., 2012).Co-immuno precipitation experiments in mycobacteria demonstrated that even though the intracellular levels of CarD and RNAP β were unchanged, $CarD^{R25E}$ and $CarD^{R47E}$ mutants co-precipitated less RNAP β than $CarD^{WT}$. These data confirmed that the R25E and R47E substitutions in CarD compromise the association with RNAP and emphasize the importance of the CarD/RNAP β1 interaction for mycobacterial growth and survival. In light of these findings, it was recommended that small molecules that interfere with the CarD/RNAP β1 interaction could be promising novel chemotherapies for killing *M. tuberculosis* (Weiss et al.,2012).

During infection, *M. tuberculosis* must withstand an arsenal of host-derived stresses, including the production of reactive oxygen species by the oxidative burst. Log-phase cultures of *M. tuberculosis* strains expressing different *carD* alleles were treated with 25 mM H_2O_2 before dilutions were plated to count the surviving CFU and found that all CarD mutants were more sensitive to H_2O_2 treatment than were the wild type, with the *M. smegmatis* $CarD^{R25E}$ mutant being more sensitive than was *M. smegmatis* expressing $CarD^{R47E}$,

demonstrating that the strength of the CarD/RNAP β1 interaction directly correlates to survival in the presence of reactive oxygen species(Weiss et al., 2012).Both *M.smegmatis* and *M.tuberculosis* strains expressing $CarD^{WT}$,$CarD^{R25E}$ (*M. smegmatis* only), or $CarD^{R47E}$ (*M. smegmatis* and *M. tuberculosis*) were transiently treated with rifampin and monitored survival by plating for CFU. In both mycobacterial species, the strength of the CarD interaction with RNAP directly correlated to survival during rifampin treatment, in that weakening the association of CarD with RNAP increases the potency of rifampin. In a similar experiment, depleting $CarD^{WT}$levels did not make *M. smegmatis* more sensitive to rifampin treatment. This implies that it is the specific interference of the CarD/RNAP interaction that compromises the bacteria's survival during rifampin treatment(Weiss et al., 2012).

It was previously known that depleting CarD increased the sensitivity of *M. smegmatis* to ciprofloxacin treatment (Stallings et al., 2009). In contrast, it was found weakening the *M. smegmatis* CarD/RNAP β1 interaction had no effect on sensitivity to 10 μg/ml ciprofloxacin treatment in 2 h of transient-treatment liquid culture assays. The finding that ciprofloxacin susceptibility is unresponsive to changes in the interaction of CarD with RNAP β1 but is sensitive to CarD protein levels indicates that CarD has a function distinct from binding RNAP. To test how CarD depletion and the CarD RID mutants would affect the sensitivity of mycobacteria to other antibiotics used to treat *M. tuberculosis* infection, transient-treatment liquid culture assays, disk zone of inhibition assays, etc were carried out.

The depletion of CarD or weakening of the CarD/RNAP β interaction had no effect on sensitivity to pyrazinamide (PZA) and no reproducible effect on isoniazid(INH) sensitivity. However,it was found that the *M. smegmatis* $CarD^{R25E}$ strain, as well as a strain depleted for $CarD^{WT}$, were significantly more sensitive to streptomycin treatment than control strains (Weiss et al., 2012). Also based on five replicate experiments it was known that the doubling time of the *M. smegmatis* $CarD^{R25E}$ mutant was 1.198 times (standard deviation, 0.067) slower than that of the wild-type control strain. Likewise, the doubling time of the *M. tuberculosis* $CarD^{R47E}$ mutant was 1.181 times (standard deviation, 0.001) slower than that of the wild-type control strain in three replicate experiments. The phenotypes associated with interfering with the CarD/RNAP β interaction were dramatically more severe in *M. tuberculosis* than in *M. smegmatis*. This was evidenced by the decrease in the growth rate of the *M. tuberculosis* $CarD^{R47E}$ mutant compared to that in the wild-type CarD-expressing strain, while in *M. smegmatis* the analogous mutant grew at wild-type rates. These findings suggest that an optimal interaction between CarD and RNAP β is more critical for the growth of *M. tuberculosis* than of *M. smegmatis, and further comparison of these bacteria may shed light on the functions of CarD* (Weiss et al., 2012).

With the crystal structure(PDB ID:4KBM) of *M.tb* CarD /RNAP β-subunit(residues 47-433) Complex, it is analysed that the CarD binding site of RNAP is located at the solvent exposed surface of the β1 domain, which is approximately 70A° away from the RNAP active site Mg+2 (based on the th EC; PDB ID: 2O5I). Despite the long distance between the binding site and the active site, this domain serves as an interaction module for various regulatory proteins, including sigma factors at different stages of transcription, and is important for RNAP -DNA binding and open complex stability (Trinh et al., 2006; Vassylyevet al., 2002). At the CarD-RNAP interface, the primary four-stranded β-sheet of the CarD N-terminal domain forms an extended eight stranded β sheet with the β1 domain of the RNAP β-subunit. Specifically, the β4 strand of CarD comprising residues Leu44 to Pro49 forms an antiparallel β sheet with the β4 strand residues Thr138 to Gln144 of RNAP β subunit. This results in a mixed β sheet topology. Surprisingly, association of RNAP with CarD results in only a 500 A°2 (otherwise solvent exposed) buried surface area, which is below average (1,500–2,000 A°2) for heteromeric protein-protein complexes (Kleanthous.,2000). While the buried surface is relatively small, it is rich in intermolecular hydrogen bonds. There are eight hydrogen bonds and 69 non bonded contacts between RNAP β subunit and CarD formed by the residues located on the β4 strands of both proteins, on the loop connecting α12 and α13 of the RNAP β1 domain, and on the turn between the β1 and β2 strands of CarD. Specifically, β1-Ile141 interacts with CarD-Arg47 (2.8A°), and β1-Ser143 interacts with CarD-Thr45 (2.7A°) through four backbone-backbone hydrogen bonds. Interestingly, the side chain-specific hydrogen bonding interactions are present only between β1-Lys142:CarD-His14 (2.9 A°),β1-Glu140:CarD-Tyr11 (2.4 A°), β1-Thr138:CarD-Asn52 (2.8 A°), and β1-Gln144:CarD-Gly42 (3.0 A°) (Gulten et al.,2013).The intermolecular interface is also stabilized by electrostatic, hydrophobic, and van der Waals interactions. In fact, electrostatic forces contribute significantly to the CarD/RNAP interaction because altering the local charge distribution at the interface was reported to abolish CarD/RNAP interaction completely (Weiss et al., 2012) But in contrast to the detail suggested by structural model generated by homology modeling (based on Tth TRCF-RID/β1 structure), mutagenesis and two-hybrid assays by Weiss et al, that β1-Glu132 interacts with both Arg25 and Arg47 directly through hydrogen bonding and that these residues are critical for

intermolecular interaction, it was observed from the Mtb CarD/RNAP structure(PDB ID:4KBM) that β1-Glu132 is not in direct contact with CarD-Arg25 and CarD-Arg47 (5.0 A° and 6.1 A°, respectively). Arg25 interacts with b1-Ile141 only through van der Waals interactions and does not appear crucial for CarD/RNAP interaction. Similarly, Glu132 and Arg47 interact only through a water molecule in the CarD/RNAP crystal structure, and Arg47 is engaged in other hydrogen bonding and van der Waals interactions and does not appear crucial for CarD/RNAP interaction. Similarly, Glu132 and Arg47 interact only through a water molecule in the CarD/RNAP crystal structure, and Arg47 is engaged in other hydrogen bonding and van der Waals interactions with β1-Ile141, β1-Glu140, and β1-Gly139. Therefore, loss of the CarD/RNAP interaction, upon E132R, R25E, and R47E mutations, should be due to these factors rather than the disruption of the direct interaction between Glu132-Arg25 and Glu132-Arg47(Gulten et al.,2013).

VII. C-TERMINAL DOMAIN

In the homology model of *M.tb* CarD built with template of *Thermus thermophilus* CarD crystal structure the CarD C-terminal domain (CarD-CTD) is a compact, all α-helical fold having 5 helices with no apparent structural similarity to any previously described fold. A notable feature of the CarD-CTD is a nearly universally conserved tryptophan (*T. thermophilus* CarD-W86) with a largely solvent exposed side chain. The tryptophan is located at the end of the CarD-CTD distal to the CarD-RID and is surrounded by a basic electrostatic surface formed from a cluster of basic residues. Within this basic surface patch, R89, R119, and R126 are universally conserved; R91 and R131 are conserved between T. thermophilus and Mycobacterium sp.; whereas K84 and R130 are found in *T. thermophilus* CarD but not Mycobacterium sp. CarD. Analysis of M. smegmatis and M. tuberculosis CarD homology models revealed that the surface-exposed tryptophan and surrounding basic surface patch are conserved features(Srivastava et al.,2013).Though on the basis of primary sequence analysis, a leucine zipper motif was proposed to be present in CarD CTD ,it was reported that no such motif was found in the crystal structure (PDB ID 4ILU) studied by kaur et al; rather CarD CTD folds into a five-helical bundle. Analysis of this helical bundle conformation using DALI server revealed that three of five helices (α2α3α4) could superimpose well on the top hits, and the remaining two helices (α1 and α5) take a unique conformation, suggesting that a five-helical bundle of CarD CTD adopts a novel five helical fold. The function of such a novel fold remains to be elucidated (Kaur et al.,2013).

But Gulten et al, with CarD/RNAP β structure complex (PDB ID:4KBM) has reported that the CarD C-terminal domain is comprised of an α-helical bundle of five α helices(α2-6) that contains an unexpected internal leucine zipper between helices α4 and α5. Helices α4 and α5 interact only through hydrophobic and van der Waals interactions, afforded by the leucine zipper. Helices α2 and α3 are positioned parallel to each other, whereas α4, α5, and α6 pack orthogonally to each other. Helices α3 and α4 are connected by a γ-turn, while α4-α5 and α5-α6 are connected by β-turns. It is also reported that the three-helix bundle of α3, α4, and α5 is involved in DNA binding. The structure of this three-helix bundle is unlike other DNA-binding proteins in the PDB. The DNA-interacting region of *M.tb* CarD is mapped to the N termini of α3 and α5, the C terminus of α4, and the β-turn connecting α4 and α5. The leucine zipper motif of CarD appears to stabilize the conformation of α4 and α5 inside the hydrophobic core and is not involved directly in dimerization or DNA interaction. Further PDBeFold, VAST, and DALI servers against the PDB and Structural Classification of Proteins database, did not identify any significant structural homologs for the C-terminal domain(Gulten et al.,2013).

Binding with DNA:

Chromatin immunoprecipitation sequencing (ChIP-seq) was used to survey the distribution of CarD throughout the M. smegmatis chromosome. Specific antibodies targeting core RNAP, σA, or a hemagglutinin(HA) epitope fused to CarD (CarD-HA) were used to co immune precipitate associated DNA that was then sequenced. It was found that CarD was never present on the genome in the absence of RNAP and was primarily associated with promoter regions and highly correlated with σA. Compilation of the ChIP-seq data and previous microarray expression profiling analyses (Stallings et al.,2009) indicated that CarD was broadly distributed on promoters of most transcription units regardless of whether they were deregulated during CarD depletion. This has lead to propose that in vivo, CarD associates with RNAP initiation complexes at most promoters and is therefore a global regulator of transcription initiation (Srivastava et al.,2013). As CarD-RID shares sequence and structural homology with the TRCF-RID, as well as a common binding mode to the RNAP β1-lobe, the crystal structure of the Thermus TRCF-RID/RNAP β1-lobe complex [Protein Data Bank (PDB) ID 3MLQ] was used as a template to generate structural model of the complex between CarD and the RNAP open promoter (RPo) complex. In the CarD/RPo model, the interaction of the CarD-RID with the RNAP β1-lobe places the CarD-CTD in a position to interact directly with the promoter DNA at the upstream edge of the −10 element on the opposite face of the DNA as σ.

Consistent with a role for the CarD-CTD in DNA interaction, CarD alone, at high concentration, is capable of non-sequence specific protein/DNA interactions, and these interactions are mediated by the CarD-CTD. The CarD-CTD/DNA interactions are centered on W86 and the surrounding basic patch, both conserved structural features of CarD. CarD potentially interacts with the DNA from about −11 to −15. The basic surface of the CarD-CTD seems to closely approach the DNA from the widened minor groove, with CarD-W86 most proximal to the DNA, roughly aligned with the −12 base pair in the open complex. To directly test the effect of CarD on rRNA promoter activity in vivo, β-galactosidase (βgal) activity was measured from lacZ which was fused to the *M. smegmatis* rrnA control region [comprising promoters rrnA-P1, -P2, and -P3 (rrnAP123)] and transformed into M. smegmatis. Depletion of CarD resulted in increased βgal activity , comparable to the increase in 16S rRNA amount observed previously during CarD depletion (Stallings et al.,2009). However, weakening the CarD/RNAP protein/protein interaction resulted in decreased βgal activity. The findings that weakening the CarD/RNAP protein/protein interaction decreases promoter activity and leads to lower 16S rRNA levels suggest that CarD may activate *M. smegmatis* rRNA transcription initiation, in contrast to the proposal that CarD acts as a repressor of rRNA transcription. It is possible that depletion of CarD,a global regulator that is essential for *M. smegmatis* viability and present at most *M. smegmatis* promoters , might give rise to pleiotropic effects that indirectly increase *M. smegmatis* rRNA promoter activity. (Srivastava et al.,2013).

VIII. DIMERIZATION

In the crystal structure PDB ID:4ILU, CarD was predicted to have two domains, where the N-terminal domain (CarD-NTD) forms an antiparallel β sheet (β1–β3) and the C-terminal domain (CarD-CTD) adopts predominantly an all-α (α1-α5) conformation. Structural and packing analyses suggested that the two monomers of CarD related by crystallographic twofold symmetry assemble to form a tight and closed homo dimer by swapping 21 N amino acids, resulting in a quasidomain-swapped (Liu et al.,2002) structure. Due to domain swapping between the β1 and β1′ strands of the monomers, the topology of the CarDNTD in the dimer constitutes (β1′β1β2β3) and (β1β1′β2′β3′) arrangements. The core of this dimeric interface includes a highly curved 14 residue long antiparallel β-sheet, formed by β1 and β1′ strands of each monomer, which contributes 18 interchain hydrogen bonds, several nonbonded contacts and a buried surface area of ~1600 A°2, as revealed by PDBe-PISA(Krissinel et al.,2007).Thus, the CarD-NTD plays an important role in dimerization. Analytical gel filtration experiments performed also found CarD to be predominantly dimeric in solution. Also it was known, *Tth* CdnLNTD, the homologue of CarD-NTD, exists as a monomer in solution, as judged by the NMR structure (Gallego-Garcia et al.,2012). On the contrary, Mxa CdnL has been reported to exist as a monomer / dimer mixture at the concentration range of 0.5–1 mM used for NMR studies(Mirassou et al.,2013). Unlike Tth CdnL-NTD or *Mxa* CdnL, monomeric *Mtb* CarD was not found in the study(Kaur et al.,2013).Structural superposition of the Mtb CarD structure on the TRCF-RID/RNAP-β1 complex (PDB ID 3MLQ; (Westblade et al.,2010)),revealed that the RNAP binding site of CarD-NTD β3 in one monomer is occluded by interactions of CarD β4′ (formed by non-native sequence) of the second monomer. Hence, suggesting that CarD crystallized in a closed conformation. These CarD-NTD β3 /CarD β4′ interactions was believed to represent a snapshot of a possible mode of CarD-NTD/RNAP-β1 interactions. The closed conformation of CarD also suggests that it is inherently flexible molecule that may undergo large domain motion to perform function(Kaur et al.,2013).

IX. SEQUENCE HOMOLOGS IN HUMANS

Blastp search done against Human protein database has given 4 hits - pantothenate kinase 2, mitochondrial isoform 1 preproprotein , plexin domain-containing protein 2 precursor, importin-9 and telomeric repeat-binding factor. These hits are with very less score and high E-value. The aligned region between the query and hits are less , with less percentage of sequence identity. This confirms the absence of highly similar proteins in humans. The hits were also analysed and found that they do not possess the domain CarD_TRCF which is present in CarD protein(Priya et al.,2012).

X. CONCLUSION

CarD protein is a bacterial transcriptional regulator and in *M.tuberculosis* is known to play a critical role in survival of the organism by downregulating few rRNA and ribosomal proteins during dormant stage in the stringent hostile environment. Also it is found to be necessary for replication and persistence during infection in mice. carD transcripts are upregulated with DNA damaging agents and also lack of CarD makes the organism more vulnerable to DNA damaging agents. This, along with CarD's ability to restore Dksa depletion strain suggests its possible role in replication. CarD is found to interact with RNA polymerase in a similar manner like TRCF, which aides in DNA strand specific repair. But it is found that TRCF would not replace CarD in that CarD might have other functions apart from RNAP interaction. Depletion of CarD confers increased sensitivity to the fluoroquinolone ciprofloxacin but is unresponsive to changes in the interaction of CarD with RNAP β1 indicates that CarD has a function distinct from binding RNAP. But it is confirmed that

mutants that weaken the CarD/RNAP interaction display an extensive antibiotic-susceptibility profile particularly to rifampin. Moreover, CarD does not have a homolog in human proteome known by sequence similarity search. This holds up the possibility of using CarD as a potential drug target in treatment against Tuberculosis. Hence presently, small molecules which can block the CarD and RNAP interaction can be promising leads against tuberculosis.

Many issues of CarD has yet to be solved. Though both *M.tuberculosis* and *M.smegmatis* show similar response in many of the experimental conditions they also differ in some responses which infers that *CarD* is essential for viability in *M.tuberculosis* but not so in *M.smegmatis.* The reason for it has to be studied in depth. Also the presence of leucine zipper motif in C-terminal domain and its role in DNA binding, is still in debate and needs to be confirmed. The function of novel fold in C-terminal domain, the dimerisation ability of CarD all need to be confirmed. Its role in sensitivity response to varied antibiotics drugs has to be studied extensively. The answers to these queries may help in designing even more efficient leads against this target.

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