Molecular dissection of the mycobacterial stringent response protein Rel

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Abstract

Latency in *Mycobacterium tuberculosis* poses a barrier in its complete eradication. Overexpression of certain genes is one of the factors that help these bacilli survive inside the host during latency. Among these genes, *rel*, which leads to the expression of Rel protein, plays an important role by synthesizing the signaling molecule ppGpp using GDP and ATP as substrates, thereby changing bacterial physiology. In Gram-negative bacteria, the protein is thought to be activated in vivo in the presence of ribosome by sensing uncharged tRNA. In the present report, we show that Rel protein from *Mycobacterium smegmatis*, which is highly homologous to *M. tuberculosis* Rel, is functional even in the absence of ribosome and uncharged tRNA. From the experiments presented here, it appears that the activity of Rel_{Msm} is regulated by the domains present at the C terminus, as the deletion of these domains results in higher synthesis activity, with little change in hydrolysis of ppGpp. However, in the presence of tRNA, though the synthesis activity of the full-length protein increases to a certain extent, the hydrolysis activity undergoes drastic reduction. Full-length Rel undergoes multimerization involving interchain disulfide bonds. The synthesis of pppGpp by the full-length protein is enhanced in the reduced environment in vitro, whereas the hydrolysis activity does not change significantly. Mutations of cysteines to serines result in monomerization with a simultaneous increase in the synthesis activity. Finally, it has been possible to identify the unique cysteine, of six present in Rel, required for tRNA-mediated synthesis of ppGpp.

Keywords: Rel protein; stringent response; ppGpp; multimerization; Rel domains

Mycobacterium tuberculosis can be categorized as one of the most successful among human pathogens as several decades of research have not yet been able to completely eradicate tuberculosis (TB), the deadly disease caused by this organism. The major barrier toward complete cure from mycobacterial infection is the unique feature, termed "latency," that these bacteria undergo on infection, leading to overexpression of genes that enable the survival of the pathogen within host organisms under oxygen- (Wayne and Hayes 1996) and nutrient-deprived (Nyka 1974) conditions. Such latent bacteria have been known to be confined in calcified lesions, termed "granulomas," which enable the dormant bacteria to resist conventional antibiotics used against active bacilli. It had been proposed that the morphology and hydrophobicity of the in vivo persistors can be mimicked in laboratory cultures by starving bacteria in vitro (Nyka 1974). Under such stress conditions, adaptation to the

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Abbreviations: pppGpp, guanosine 3′-diphosphate 5′-triphosphate; ppGpp, guanosine 3′, 5′-bis(diphosphate); IPTG, isopropyl β-D-1-thiogalactopyranoside; Ni-NTA, nickel-nitrilotriacetic acid; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; PEI, poly(ethyleneimine); TLC, thin layer chromatography; DTT, dithiothreitol; TCA, trichloroacetic acid; kb, kilobases; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; MALDI, matrix-assisted laser desorption and ionization.

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environment plays a critical role in the survival of the organism. A complete study of the survival strategies used by mycobacteria under starvation will aid in successful control of the growth and persistence of these organisms (Chatterji and Ojha 2001).

One of the important adaptive responses that bacteria display is the "stringent response," which is typically characterized by down-regulation of rRNA synthesis and up-regulation of protein degradation and amino acid biosynthesis (Cashel et al. 1996; Paul et al. 2004). These responses are mediated by stringent factors, namely, pppGpp and/or ppGpp, which are synthesized by the transfer of PPi moiety from ATP to the 3′ end of GTP or GDP, respectively (Avarbock et al. 2000; Chatterji and Ojha 2001). The functional significance of (p)ppGpp has been studied extensively (Svitil et al. 1993; Garza et al. 2000; Sun et al. 2001). In earlier studies, we have reported that under nutrient starvation, *M. smegmatis* shows accumulation of the stringent factor ppGpp (Ojha et al. 2000), thereby indicating a link between persistors and the stringent response. It has also been demonstrated that in *M. tuberculosis* strains wherein the gene required for (p)ppGpp synthesis, *rel*, is deleted, the long-time survival of the pathogen is remarkably compromised in growing cultures (Primm et al. 2000) and in animal models (Dahl et al. 2003). In a recent study, the persistence of *M. tuberculosis* within host granulomas has been directly linked with the *rel* gene (Karakousis et al. 2004). In *M. smegmatis*, the deletion of *rel* causes changes in cellular and colony morphology (Dahl et al. 2005) and an altered association of mycobacteria with cultured macrophages (Mathew et al. 2004).

In *Escherichia coli* and several Gram-negative bacteria, two genes, namely *relA* and *spoT*, maintain cellular (p)ppGpp levels with their protein products, RelA, carrying out (p)ppGpp synthesis, and SpoT, required for hydrolysis, respectively (Xiao et al. 1991). In *M. tuberculosis* and other Gram-positive organisms, a single bifunctional protein, Rel, carries out both synthesis and hydrolysis of (p)ppGpp, thereby regulating its levels within the cell (Avarbock et al. 1999, 2000). Purified Rel_{Mtb} shows (p)ppGpp synthesis in vitro in a ribosome-independent manner (Avarbock et al. 1999). In an interesting study with *Streptococcus equisimilis*, it has been proposed that in the bifunctional Rel protein, ppGpp hydrolysis and synthesis activities are maintained by an interaction between the N-terminal and the C-terminal domains of the protein (Mechold et al. 2002). The molecular structure of the N-terminal domain of Rel from *S. dysgalactiae equisimilis*, solved using X-ray crystallography, further supports the two-domain cross-talk model for the synthesis and degradation of ppGpp (Hogg et al. 2004). This "intramolecular cross-talk" is proposed to be necessary for regulation of the two antagonistic activities. It appears that at any given time only one activity is present in the protein, as it will prevent simultaneous synthesis and hydrolysis of ppGpp and unnecessary consumption of ATP.

When Rel protein sequence (Rel_{Msm}, 797 amino acids)—as obtained by carrying out BLAST search at TIGR CMR database (http://www.tigrblast.tigr.org/cmr-blast/) in the *M. smegmatis* genome using Rel protein sequence from *M. tuberculosis* as template—was submitted to the National Center for Biotechnology Information (NCBI) Web site (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) for domain search using Conserved Domain Database (Marchler-Bauer et al. 2005), several interesting properties emerged. The protein was found to be composed of different domains, and it appeared that various domains present in fairly large Rel protein might have distinct functions with interdependence. Thus, we were tempted to analyze these domains separately for their (p)ppGpp synthesis and hydrolysis activities.

During the course of our studies, we observed multimerization of wild-type Rel protein. In a recent work on Rel_{Mtb}, it has been suggested that the protein forms trimer by strong noncovalent interactions and disrupts to monomer upon addition of the substrate. In addition, the trimer state is considered to be less active as compared with monomer (Avarbock et al. 2005). Our studies were suggestive of covalent interactions mediated by free thiols of several cysteines present in the protein as responsible for the various multimers obtained. Cysteine mutants have therefore been examined toward their possible role in regulation of protein activity.

Results

Rel_{Msm} protein consists of several domains as obtained from Conserved Domain Database search and shows a high homology with other proteins of this family

The results shown in Figure 1A clearly indicate that the protein is composed of four domains with the following functions: HD (amino acids 110–239) is a metal dependent phophohydrolase domain (Pfam accession no. PF01966 [Aravind and Koonin 1998]); RSD domain (amino acids 300–412) stands for the RelA_SpoT domain and represents the RelA_SpoT region found in RelA/SpoT proteins (Pfam accession no. PF04607). In *M. smegmatis* (this study), *M. tuberculosis* (Avarbock et al. 2005), and *S. equisimilis* (Hogg et al. 2004), these domains are present at the N terminus and harbor the catalytic site. TGS (Pfam accession no. PF02824; amino acids 458–518), named after ThrRS (Threonyl tRNA synthetase), GTPase, and SpoT, is suggested to have a regulatory role with ligand binding ability (Sankaranarayanan et al. 1999); ACT domain (Pfam accession no. PF01842; amino acids 720–793) is widely present in proteins that undergo regulation by cellular amino acid levels (Schuller et al. 1995; Chipman and Shaanan 2001;

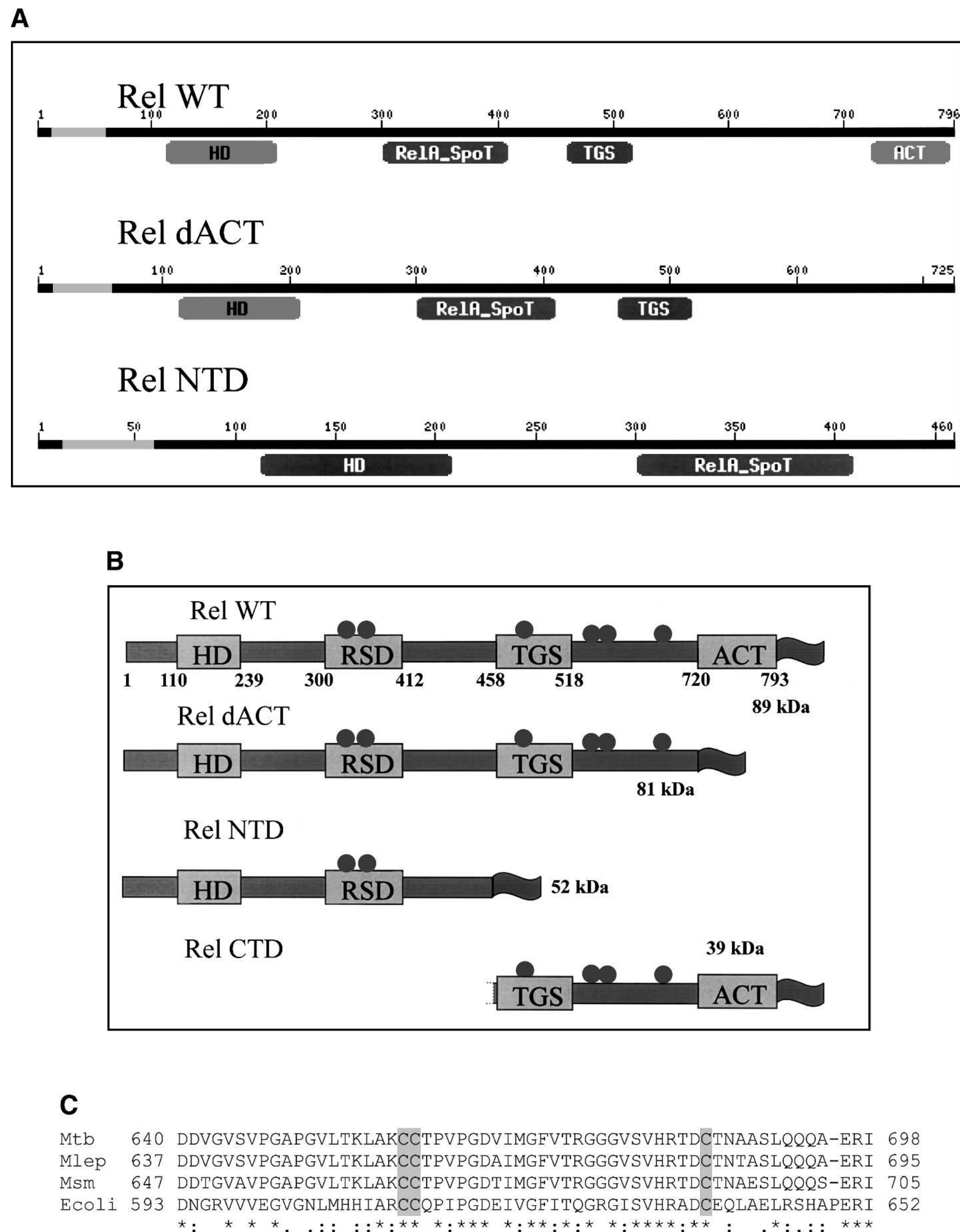

Figure 1. Database search for the identification of conserved domains and cysteines. (*A*) Domain prediction in Rel WT protein from *M. smegmatis* and in its deleted versions. (*B*) Deletion in the Rel full-length protein based on the prediction of the domains in Rel WT. The molecular masses of the constructed fragments are also mentioned. The region at the *end* of each bar represents the Hexa-histidine tag. Positions of the start and the end of the domains have been mentioned. The locations of the cysteines present in all of the constructs have been marked by filled circles (●). (*C*) Sequence alignment of Rel protein sequences from *M. tuberculosis*, *M. leprae*, *M. smegmatis*, and *E. coli*. Only the C-terminal region with three conserved cysteines is shown.

Liberles et al. 2005). Both TGS and ACT domains are present at the C-terminal half of Rel_{Msm} and Rel_{Seq}, wherein a regulatory role for these domains has been suggested in the latter (Mechold et al. 2002).

Rel_{Msm} shows a high homology with Rel_{Mtb} and Rel_{Mlep}, (sequences obtained from their respective genomes at NCBI at http://www.ncbi.nlm.nih.gov/), the identities being 88.3% and 86.4%, respectively. We found that the six cysteines present in Rel_{Msm} (Fig. 1B) are conserved in all of the other Rel proteins present in Gram +ve organisms considered for multiple sequence alignment. Only three such C-terminally located cysteines have been shown (Fig. 1C). In *E. coli* RelA, a Gram –ve bacteria, though the identity with Rel_{Msm} was found to be only 34.3%, the cysteines present in the

C-terminal region were still conserved (Fig. 1C). This is immediately suggestive of an important role played by cysteine residues in this protein.

Rel full-length protein and its deleted versions are active both in vitro and in vivo

The regulation of Rel protein is a subject of active study due to its direct involvement in stringent response (Cashel et al. 1996; Primm et al. 2000) as well as due to its bifunctional nature (Avarbock et al. 2000, 2005; Mechold et al. 2002; Hogg et al. 2004). We have also noticed interesting features of its promoter element and transcription start site (Jain et al. 2005). In order to study its biochemical properties, the *rel* gene from *M. smegmatis* was PCR amplified and cloned in pET21b vector, thereby gaining a hexa-histidine tag at the C terminus to ease purification. The protein was expressed in *E. coli* BL21 (DE3) cells by induction with IPTG and purified to homogeneity as judged by a SDS-PAGE profile (Fig. 2, lane c) and mass spectrometric analysis. The purified protein showed pppGpp synthesis activity in vitro even in the absence of uncharged tRNA and ribosome (Fig. 3A, lane b). It has been reported earlier (Haseltine and Block 1973) that in *E. coli*, purified RelA does not synthesize stringent factor in the absence of tRNA or ribosome. Our result clearly shows that this is not the case with a Gram +ve organism like *M. smegmatis*. Similar results have also been obtained in the case of Rel_{Mtb} (Avarbock et al. 1999, Avarbock 2000). In order to further confirm this, we compared the synthesis activity of Rel protein in the presence and absence of crude cell extract. No significant difference was observed in the activity of Rel protein in the absence or the presence of the crude cell lysate (Fig. 3A, lanes b and c, respectively), indicating that the protein is functional and other factors are redundant. The cell extract alone was used as a negative control (Fig. 3A, lane a).

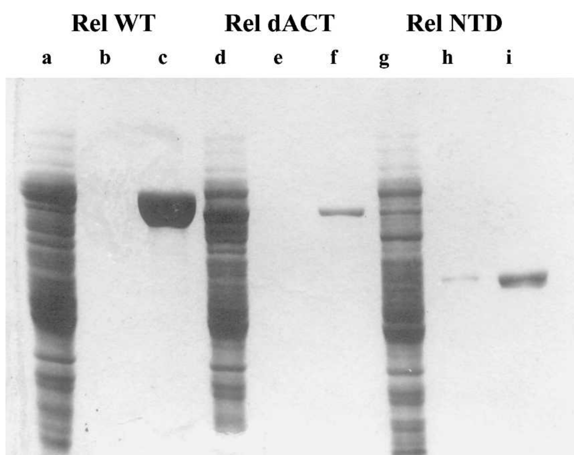

Figure 2. Eight percent of SDS–polyacrylamide gel showing the purification of Rel WT protein (lanes *a-c*) and its deleted versions. (Lanes *d-f*) Rel dACT, (lanes *g-i*) Rel NTD. Lanes *a*, *d*, and *g* represent the crude cell lysate of the respective proteins, whereas lanes *c*, *f*, and *i* represent the purified proteins. Lanes *b*, *e*, and *h* are column wash, showing no bands due to the large volume (100 column volumes) and low protein concentration.

In order to ascertain the phosphohydrolase activity of the HD domain, synthesis activity of the RSD domain and the regulation of pppGpp synthesis by a C-terminal end (both TGS and ACT domains), we made several deletion constructs that were all terminally attached to hexa-histidine tag. First, a deletion from the C terminus was made such that the ACT domain is deleted, thereby giving rise to Rel dACT (Fig. 1B). Secondly, both the ACT and the TGS domains were truncated from the protein, giving Rel NTD (Fig. 1B). The proteins were purified from these constructs in a similar way as for the Rel WT protein (Fig. 2, lanes f,i). We observed that under in vitro assay conditions, both Rel dACT and Rel NTD proteins were active (Fig. 3B). Interestingly, Rel NTD showed a much higher pppGpp synthesis as compared with Rel dACT and the full-length Rel WT protein. This observation confirms the regulatory role suggested for the TGS domain. It can be proposed that the TGS domain alone, or in association with ACT, regulates Rel_{Msm} protein activity. Specific activities obtained for each of these proteins for pppGpp synthesis are shown in Figure 3C.

Keeping in mind the role of uncharged tRNA in pppGpp synthesis by RelA, we studied the effect of tRNA in vitro on pppGpp synthesis by Rel or its different deleted mutants (Fig. 4). In Rel WT, the presence of tRNA increased the synthesis of pppGpp by ~1.5-fold, which was comparable to the enhancement observed in Rel_{Mtb} (Avarbock et al. 2000). In Rel NTD, where the C-terminal half was deleted, even high concentrations (40 μg) of tRNA did not show any effect on the synthesis activity (Fig. 4). This observation is suggestive of interaction of tRNA with the C terminus.

Similarly, we checked the hydrolysis of pppGpp in the presence of Rel WT and Rel NTD proteins. We found that the hydrolysis activity of the two proteins did not change significantly (Table 1). However, in the presence of tRNA, Rel WT showed a drastic reduction in the hydrolysis, whereas Rel NTD remained largely unaffected. This further supports our finding that tRNA interacts with the Rel C-terminal to modulate its synthesis and hydrolysis activities. It is worth mentioning that in the case of Rel_{Mtb}, the K_m for pppGpp and ppGpp in hydrolysis assays was shown to increase in the presence of uncharged tRNA as compared with the basal level, thus reducing the protein's hydrolysis activity (Avarbock et al. 2000).

Circular dichroism study of full-length Rel and Rel mutants

Although the activity of the Rel NTD was higher than the Rel WT protein, we observed that both proteins showed

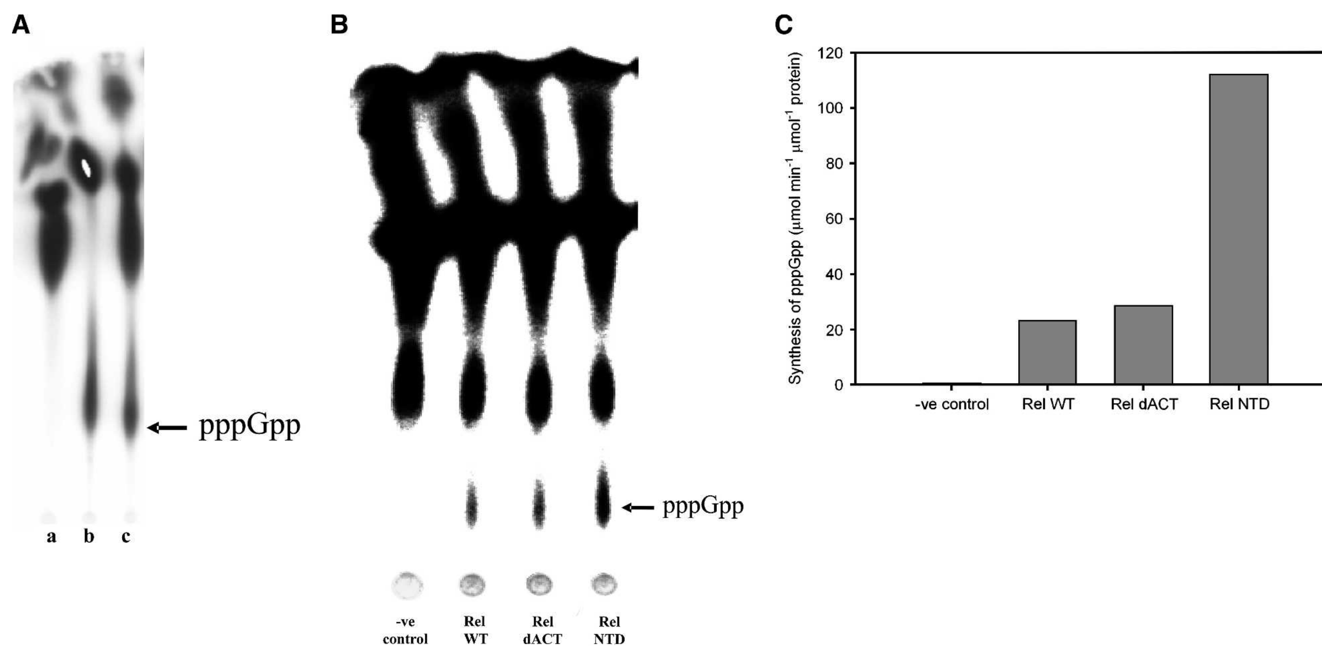

Figure 3. In vitro synthesis activity of Rel WT protein and its variants. (*A*) pppGpp synthetic activity assay with the purified Rel WT protein. Lane *b* shows the synthesis of pppGpp by purified Rel WT protein. Lane *c* represents the activity assay with the Rel WT-purified protein mixed with the crude cell lysate of *E. coli* BL21 (DE3), which alone does not give any activity (lane *a*). (*B*) pppGpp synthetic activity assay with the 5-μM purified Rel WT, Rel dACT, and Rel NTD proteins, showing that all of the three proteins are active in vitro. Reaction mixture without any protein was used as –ve control. (*C*) Specific activity measurement of the Rel WT, Rel dACT, and Rel NTD proteins and the negative control.

similar secondary structure profile with a predominant α-helical nature as measured by CD spectroscopy (Fig. 5A). This is in accordance with the crystal structure data of Rel_{Seq}. When the C-terminal half of the protein (453–797) was subsequently cloned in pET21b vector and expressed, the purified protein showed a CD profile suggestive of a partially unstructured protein (Fig. 5A). We would like to mention here that Rel CTD upon elution aggregated due to the presence of many cysteine residues and required a reducing environment to prevent this aggregation. The reason for this, we hypothesize, is that the cysteines present in the Rel CTD are exposed to the solvent due to the partial unstructured nature of the protein. The thiols of these cysteines, in the absence of reducing agents and under the mild basic conditions used for purifications, are available for intermolecular disulfide bond formation. These intermolecular disulfides can be obtained only in partially or completely unstructured proteins and cannot be argued by cysteine exposure to the solvent due to regular breathing movement in the proteins. To further confirm the partial unstructured property of Rel CTD, the protein was incubated with different concentrations of urea for 5 h at 25°C, and CD spectrum at 222 nm was recorded. Rel NTD was used here as control. We observed that the denaturation

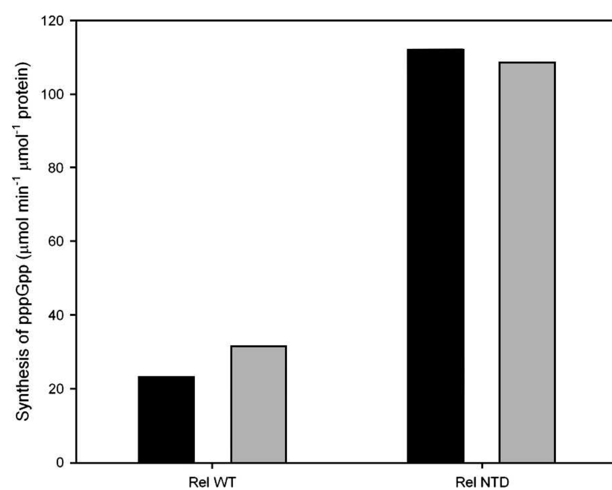

Figure 4. Effect of 40 μg uncharged tRNA on the synthesis activity of Rel WT and Rel NTD proteins. Black bars represent the absence of tRNA, whereas gray bars represent its presence.

Table 1. *Specific activities for hydrolysis of pppGpp by Rel WT and Rel NTD in the presence and the absence of 40 μg tRNA*

	Specific activity (mol GTP mol^{-1} protein min^{-1})	
Protein	−tRNA	+tRNA
Rel WT	1.02 ± 0.02	0.21 ± 0.04
Rel NTD	1.16 ± 0.04	1.06 ± 0.03

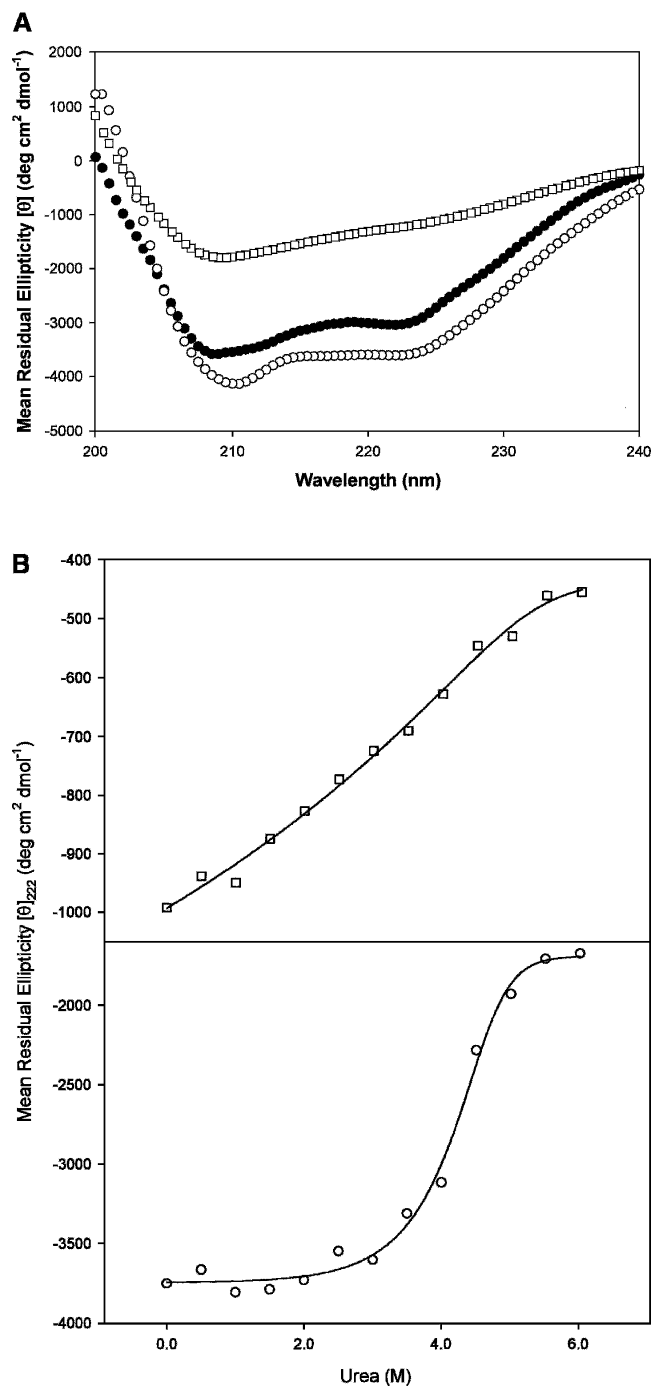

Figure 5. (*A*) Circular dichroism spectra of Rel WT (●) compared with that of Rel NTD (○) and Rel CTD protein (□), showing that the Rel CTD protein is largely unstructured. The *Y*-axis represents the mean residual ellipticity. (*B*) Urea denaturation profiles of Rel CTD (□) and Rel NTD (○). The proteins were denatured with increasing concentrations of urea. The *Y*-axis represents the mean residual ellipticity at 222 nm.

profile of Rel CTD was suggestive of a noncooperative denaturation as compared with that of Rel NTD, which showed cooperative unfolding (Fig. 5B). Each point in the graph represents five accumulations after substracting the contribution from buffer.

The quantitation of %Helix, %Sheet, and %unordered was carried out using the programs CDSSTR (Compton and Johnson 1986; Sreerama and Woody 2000) and CONTINLL (Provencher and Glockner 1981; van Stokkum et al. 1990) using two different protein data sets, SP43 and SDP48. Both of the data sets gave similar results, and these matched closely with the values obtained using the other program. In the case of Rel WT protein, the values obtained using CDSSTR program were 70.4% helix, 17.5% sheet, and 12.6% unordered, whereas Rel NTD gave 70.2% helix, 16% sheet, and 13% unordered. On the other hand, Rel CTD showed 56.7% helix, 11% sheet, and 31.6% unordered. These data, although having been obtained after comparing the CD spectra of the proteins with the standards and showing very low RMSD, should be considered with some reservation. However, when we estimated the structural parameters for Rel_{Seq} (PDB ID 1VJ7; Hogg et al. 2004) using the program PROMOTIF (Hutchinson and Thornton 1996), the values matched to a great extent with the data obtained in the case of RelNTD.

In vivo activity of Rel and its variants

Next, our attempt was to find out whether the full-length and the deleted versions of Rel protein were active under in vivo conditions. All of the proteins were cloned in a *Mycobacterium–E. coli* shuttle vector pMV261 under *hsp60* promoter (Stover et al. 1991). The constructs were transformed in *M. smegmatis* Δ*rel* strain (Mathew et al. 2004) in order to rule out any possible synthesis from the genomic copy of the *rel* gene. Cells were grown in the presence of $H_3{}^{32}PO_4$ to label all of the phosphates in the cell. Labeled cells were harvested and lysed, and the cleared lysate was run on PEI-coated TLC to check for the appearance of the (p)ppGpp spot. It was observed that in all of the cases viz. Rel WT, Rel dACT, and Rel NTD, the "alarmone" molecule (p)ppGpp was synthesized (Fig. 6), thus indicating that the proteins were active in vivo. The mobility of the spot was confirmed by simultaneously running purified pppGpp and GTP as standards. *M. smegmatis* Δ*rel* strain transformed with empty pMV261 plasmid was used as a negative control. In this experiment we observed a higher spot density for (p)ppGpp in the case of Rel WT as compared with Rel NTD. This was because of the fact that strain containing Rel NTD grew slowly, and therefore the total cell mass used in the assay could not be normalized.

RSD and CTD domains of Rel_{Msm} have no activity in isolation

When RSD domain was cloned and expressed, the protein was found to be present in inclusion bodies, and therefore

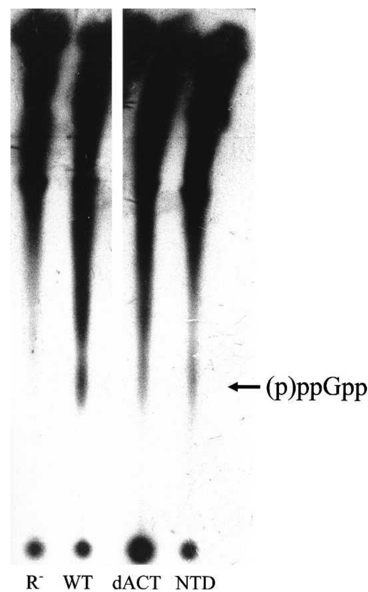

Figure 6. In vivo (*p*)ppGpp synthesis activity assays with the Rel WT, Rel dACT, and Rel NTD proteins complemented in Δ*rel* strain of *M. smegmatis* with a *Mycobacterium–E. coli* shuttle vector pMV261. *M. smegmatis* Δ*rel* strain having pMV261 plasmid was used as negative control (R^-).

it was purified after solubilizing in urea. Upon refolding by dialysis, no pppGpp synthesis activity was found to be lacking in this protein. We could not infer here whether the inactivity was due to incomplete refolding or whether the presence of HD domain was a prerequisite for RSD activity. However, the crystal structure of Rel_{Seq} suggested strong interactions between the HD and RSD domains and the presence of the substrate in either of the two sites to be the deciding entity for the kind of activity (synthesis/hydrolysis) the enzyme would perform (Hogg et al. 2004). In our experiment, the interaction between the HD and RSD domains has been disrupted upon construction of the deletion mutant, and this could possibly have resulted in the loss of activity. In a recent study on Rel_{Mtb} protein, a small construct of 87–394 amino acids showed the synthesis and not the hydrolysis activity (Avarbock et al. 2005). This construct, although containing the entire RSD domain, still retained a substantial part of the HD domain (~70 amino acids). Furthermore, in the same study, yet another construct 1–156 amino acids did not have any RSD domain and also showed no detectable hydrolysis activity.

Rel CTD protein did not show any activity in vitro because of the absence of catalytic domains. We further tried to find out whether Rel CTD could complement Rel NTD for the loss of the C-terminal in this protein, and thus bring back the regulation. No difference in the pppGpp synthesis was observed in the case of Rel NTD alone as compared with Rel NTD complemented with increasing concentrations of Rel CTD (data not shown). This suggests that the two proteins do not show high affinity to each other when present in isolation. It must be noted here that the assay buffer already contained 1 mM DTT in order to keep the protein in a nonaggregated state. The association of the N- and C-terminal domains is also probably driven by other accessory cellular factors, such as tRNA that binds to one of the domains, causing a conformational change, thereby resulting in "interdomain cross-talk." It should also be noted here that the C-terminal half is largely unstructured and the binding of accessory ligands probably induces structure in the TGS and ACT domains, thereby promoting domain–domain interaction.

Rel WT protein oligomerizes in vitro, but monomer is active in pppGpp synthesis

Rel WT protein, on a denaturing polyacrylamide gel, in the absence of reducing agents such as β-mercaptoethanol or dithiothreitol, formed multimers with a heterogenous distribution (Fig. 7A). There appeared several species, including the one that could not enter the gel. However, a dimer form could be established unambiguously by comparison of the movement of protein on the gel along with standard molecular weight markers. The oligomerization due to disulfide bond formation appeared to be a possibility, as upon running the protein on a denaturing gel after boiling in the presence of SDS (2.0%) and in the absence or the presence of 100 mM DTT, multimeric forms were retained in the former case, whereas in the latter, a single band corresponding to monomeric Rel could be seen (Fig. 7A). It also showed that the appearance of higher molecular weight bands was not due to noncovalent interactions. To further prove that multimerization was a result of intermolecular disulfide bond, a two-dimensional analysis of this protein was performed, in which the protein was run in the first dimension on a denaturing gel in the absence of DTT and then in the second dimension again on a denaturing gel after incubating the gel strip in 100 mM DTT. All higher oligomers that were observed in the first dimension appeared at the same position in the second dimension, thus indicating that all of the higher forms, including the one that remained in the well, were composed of Rel protein (Fig. 7B). It further confirmed that the protein formed higher molecular weight species by interprotein disulfide bonds. The multimerization status of the proteins was further confirmed by gel-filtration chromatography using a Superdex 200 10/300 GL column in the presence of 6 M urea with or without 10 mM DTT. The graph obtained showed the mobilities corresponding to dimer and monomer (data not shown).

We then tried to find out the minimum concentration of DTT required for converting multimers to monomer.

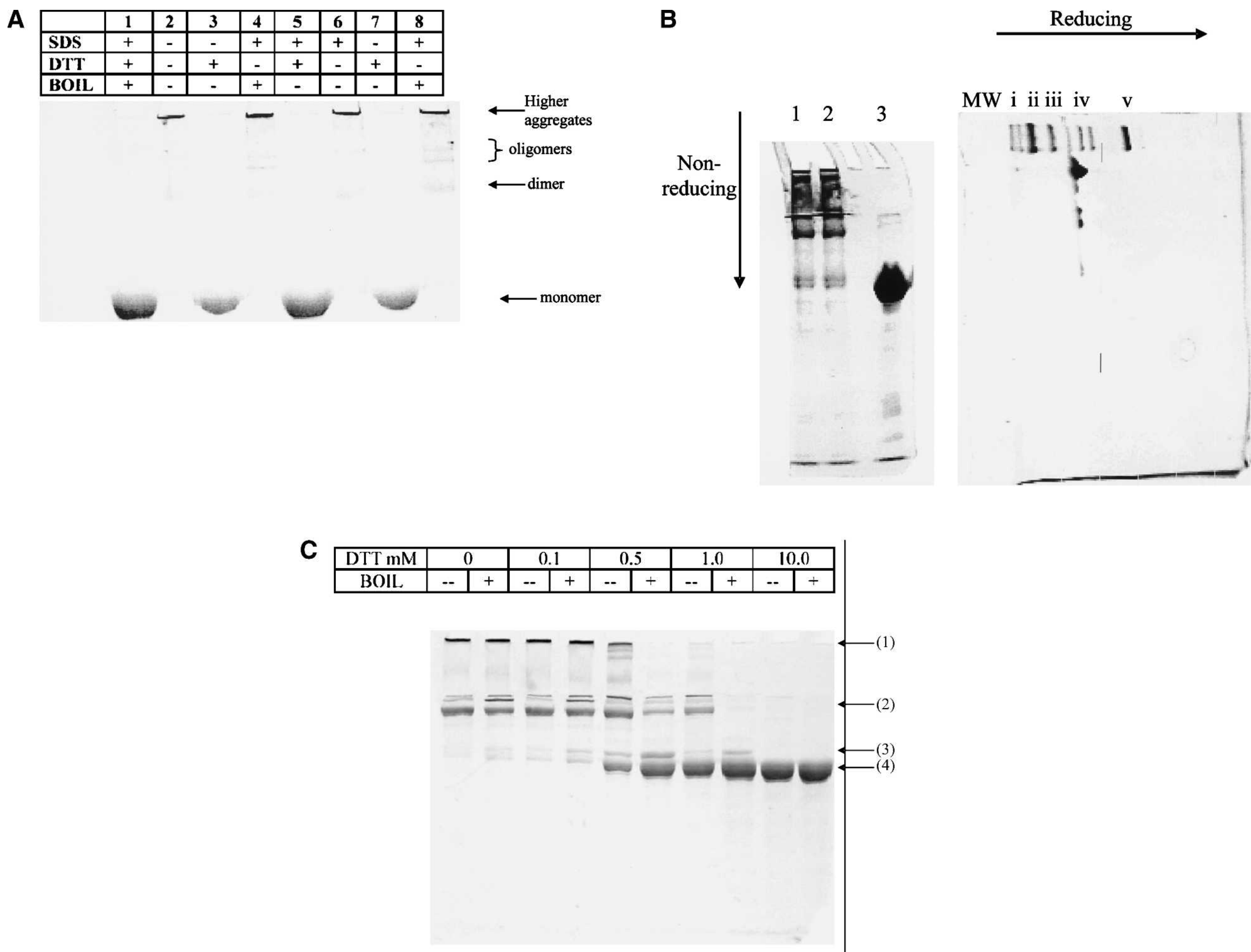

Figure 7. (*A*) Multimerization of Rel WT protein. The protein was run on an 8% SDS polyacrylamide gel under the conditions mentioned *above* each lane. Three conditions viz. SDS (2%), DTT (100 mM), and boiling were taken to run the protein. ''+'' represents the presence of that condition, whereas ''-'' represents the absence. (*B*) Two-dimensional analysis of Rel WT protein. In the first dimension, the protein was run in a nonreducing condition on an 8% denaturing polyacrylamide gel with modified loading buffer (see Materials and Methods). Lanes *1* and *2* represent the Rel WT protein run in nonreducing condition; lane *3* represents the same run in reducing condition. One lane similar to lanes *1* and *2* was cut and loaded on the second dimension (8% denaturing polyacrylamide gel) after incubating with the buffer as mentioned in Materials and Methods. High molecular weight marker (Amersham Biosciences) is also loaded alongside to confirm the mobility (MW markers [kDa] *i* = 220, *ii* = 170, *iii* = 116, *iv* = 76, *v* = 53). (*C*) Multimerization of Rel is dependent on DTT concentration. Rel WT protein was loaded with or without boiling after treatment with the indicated concentrations of DTT on an 8% denaturing polyacrylamide gel. Different species (1, 2, 3, and 4) of Rel WT protein have been pointed out by arrows. Arrow indicating ''1'' corresponds to the aggregated species of Rel WT protein that does not enter the gel;''2'' represents the dimer and oligomers; ''3'' and ''4'' are the monomers. ''3'' probably is due to the intramolecular disulfide bond formation or cysteine oxidation.

Different concentrations of DTT as indicated in Figure 7C were used with or without boiling Rel WT (20 μg) protein samples. In all cases, the SDS concentration was kept constant at 2.0%. We found that DTT at a concentration of 10 mM was sufficient to completely monomerize the protein even without boiling. Rel dACT, with all six cysteines, showed similar multimerization like Rel WT, whereas Rel NTD with only two cysteines did not multimerize at all (data not shown). Thus we were tempted to conclude that the C-terminal four cysteines (C492, C666, C667, and C692) were responsible for multimerization. We further investigated whether multimers of Rel were the active species for pppGpp synthesis. An activity assay profile at different DTT concentration like that in Figure 7C showed that the pppGpp synthesis significantly increased with increasing DTT, saturating at 10 mM concentration (data not shown). Rel NTD, on the other hand, did not show any DTT dependence for pppGpp synthesis.

That the monomer of Rel is also the functional unit in vivo was further proved by Western analysis. We used

here *E. coli* BL21 (DE3) cells overexpressing Rel WT protein. Antibodies against the Rel WT protein and the hexa-histidine tag were used to carry out the Western analysis of the crude cell lysate. It was observed that the Rel WT protein, upon overexpression in *E. coli*, did not form multimers (Fig. 8A,B). This result is not unexpected because of the fact that the reducing environment of the cell leads to reduction of solvent-accessible protein disulfides.

The hydrolysis activity of the full-length protein, on the other hand, remained unchanged with high concentrations of DTT (Table 2). We at present do not have a convincing explanation for this observation, but it is probably due to the fact that the HD domain, required for hydrolysis, is situated at the extreme N terminus and even in multimeric forms is still available for pppGpp hydrolysis.

Cysteine mutagenesis renders the protein monomer—The bifunctional property of the enzyme changes as a function of mutation

As our observations suggested that the multimerization in Rel protein is due to intermolecular disulfide bond formation, mutation in one or a few of the cysteines responsible for multimerization should convert the protein into a monomer. After mutating all of the four cysteines (C492, C666, C667, and C692), the protein was largely present in the form of a monomer as judged by the absence of a higher molecular weight species on the SDS-PAGE run in the absence of a reducing agent (Fig. 9A, lane e). We noticed that the native Rel protein upon multimerization showed several different forms, which migrated inside the gel (8%), and a significant fraction remained in the well due to extensive cross-linking (Fig. 7C). On mutating C666 and C667 to serines, although the protein did not aggregate, higher multimers were still present, as in the case of Rel WT (Fig. 9A, lanes a,b). Next, the nature of these multimers moving inside the gel was investigated. We decided to mutate three cysteines at a time, generating two sets, and retaining just one cysteine of the four cysteines at the C-terminal end. In this way we created one set with C492S, C666S, and C667S mutations, and C666S, C667S, and C692S as the other set. Both of these sets showed two different bands (Fig. 9A, lanes c and d, respectively) matching with a higher doublet of lanes a and b. Both of these bands are presumed to be dimers with different mobilities, as in both cases there was only one cysteine left in the C-terminal end of the mutant Rel. We have already shown that the other two cysteines at the N-terminal half do not participate in cysteine-dependent multimerization. These observations were further confirmed by Western blot analysis with the mutant proteins (Fig. 9B).

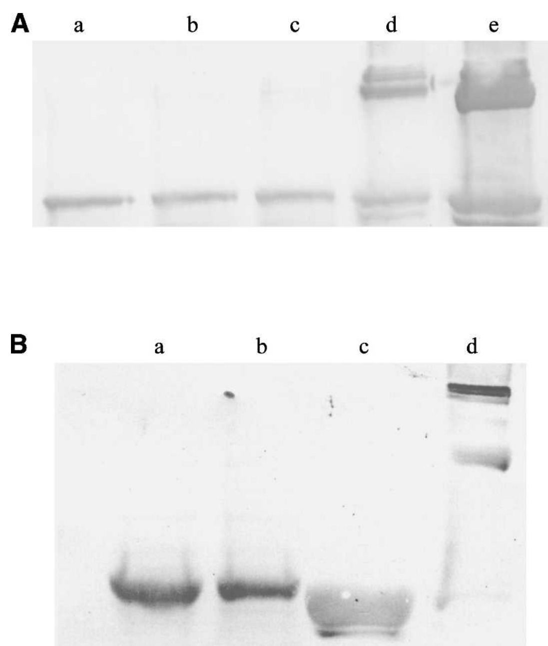

Figure 8. Western analysis of the Rel WT protein expressed in *E. coli* BL21 (DE3). (*A*) Rel WT protein probed with anti-Rel antibodies. Lanes *a–c* represent the crude cell lysate overexpressing Rel protein and treated as mentioned in Materials and Methods. Lanes *d* and *e* are the protein (control) run without DTT. (*B*) Rel WT protein probed with anti-His tag antibodies. Lanes *a* and *b* represent the crude cell lysate treated as mentioned in Materials and Methods. Lanes *c* and *d* represent the protein run in the presence and the absence of DTT, respectively, and act as controls.

Table 2. *Specific activities for hydrolysis of pppGpp by Rel WT and Rel NTD in the presence and the absence of 10 mM dithiothreitol*

Protein	Specific activity (mol GTP mol^{-1} protein min^{-1}) −DTT	+DTT
Rel WT	1.01 ± 0.04	1.03 ± 0.02
Rel NTD	1.18 ± 0.07	1.15 ± 0.09

The synthesis and the hydrolysis activities of these mutants were assayed, and it was observed that the synthesis activity of the mutant proteins increased as a function of mutation (Fig. 10), but hydrolysis activity remained largely unchanged (data not shown). In addition, we observed that all of the mutants except one, where all of the four cysteines were mutated to serines, showed increased pppGpp synthesis in the presence of 10 mM DTT, as expected (Fig. 10).

C692S makes synthesis activity of the full-length protein nonresponsive to tRNA

With the cysteine mutants thus constructed, we tried to observe the effect of tRNA on pppGpp synthesis. It was shown before (Fig. 4) that Rel NTD not only yielded

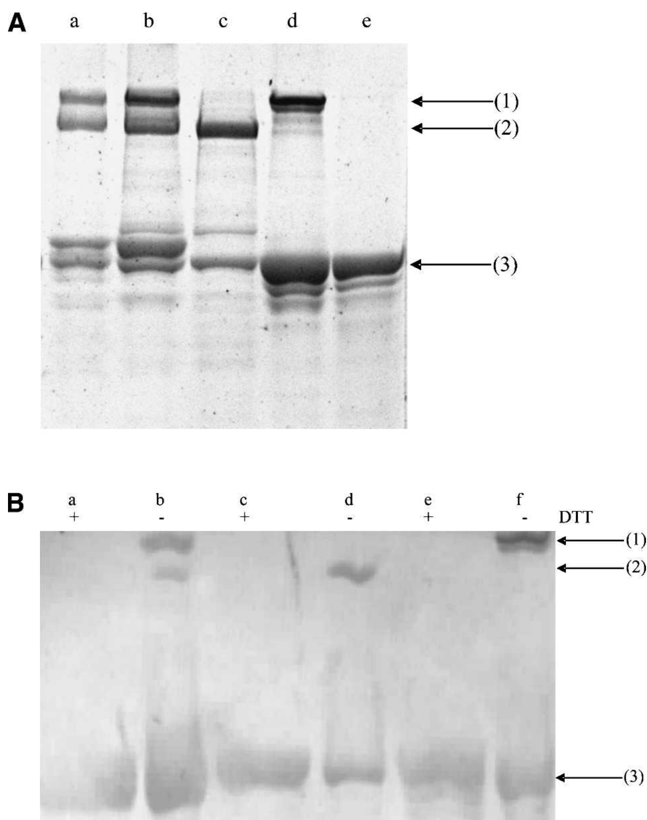

Figure 9. (*A*) An 8% SDS-PAGE showing Rel full-length protein with different cysteine mutants. (Lane *a*) Rel WT; (lane *b*) Rel C666S, C667S; (lane *c*) Rel C492S, C666S, C667S; (lane *d*) Rel C666S, C667S, C692S; (lane *e*) Rel C492S, C666S, C667S, C692S. All of the proteins were run under denaturing conditions in the absence of DTT. Arrows at 1 and 2 indicate the position of dimers with different mobilities. Arrow at 3 indicates the mobility of monomer. (*B*) Western analysis of the cysteine mutants. The proteins were run in the presence and the absence of DTT. (Lanes *a*, *b*) Rel C666S, C667S; (lanes *c*, *d*) Rel C492S, C666S, C667S; (lanes *e*, *f*) Rel C666S, C667S, C692S. "+" represents the presence of DTT, whereas "−" represents its absence. Arrows at 1 and 2 indicate the position of dimers with different mobilities. Arrow at 3 indicates the mobility of monomer.

higher pppGpp synthetic activity compared with native Rel but the effect of 40 μg of tRNA was also minimal in the former case. Similarly, we observed that only two cysteine mutants (Rel C666S, C667S and Rel C492S, C666S, and C667S) resulted in a stimulatory effect of tRNA, whereas Rel C666S, C667S, C692S and Rel C492S, C666S, C667S, and C692S remained unaffected (Fig. 11). Thus, by process of elimination, the results indicated that the C692S in Rel is required for tRNA-mediated synthesis of pppGpp in vitro.

Discussion

Several studies are underway to completely characterize the dormant stage of *Mycobacterium* toward attempts at developing efficient drugs that target this stage of the bacterium in human hosts. The present study is one such analysis wherein one of the proteins that plays a crucial role for starvation response in mycobacteria has been characterized. Rel protein from *M. smegmatis* has been cloned, expressed, and characterized using a series of systematic deletions and mutants under different conditions. The Rel_{Msm} is very similar to Rel_{Mtb} and is composed of four domains, as revealed by the Conserved Domain Database search at NCBI. HD and RSD domains that are situated in the N-terminal half of the protein have a catalytic role to play, as confirmed by the bifunctional nature observed in the Rel NTD protein. We find by sequence alignment that Rel_{Msm} shares a high homology with other proteins of this family, especially with Gram +ve bacteria. With Gram –ve bacteria, however, the identity is not very high, but certain residues—for instance, cysteines—are found to be conserved. We have further mutated these cysteines to find the effect of these residues on the protein activity and stability. The enzymatic activity of a protein is a cumulative effect of a well-formed active site as well as the spatially important proximal residues. However, basal protein function can indeed be measured with a minimal sequence element using domainwise mapping, although in this method, the contribution of residues from neighboring domains no longer exists. Such domainwise mapping of different active sites has been reported before for several other proteins (Hayward et al. 1991; Igarashi and Ishihama 1991; Blatter et al. 1994; Kim et al. 1997; Lipari et al. 2001; Lee et al. 2002; Young et al. 2004).

We show here that Rel WT protein is active in vitro and the activity is independent of ribosome and uncharged

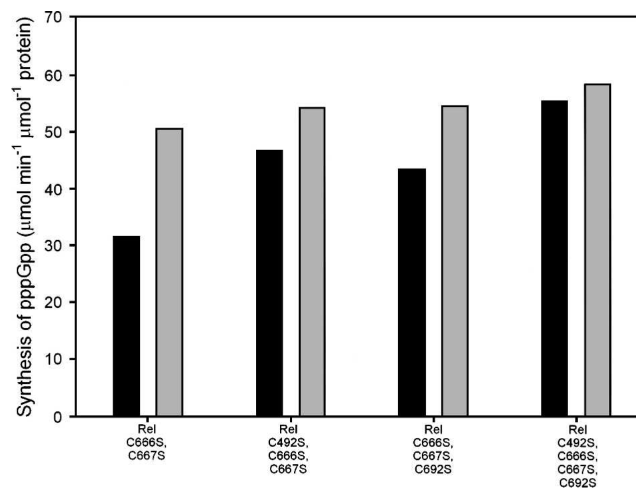

Figure 10. Effect of 10 mM DTT on the synthesis activity of Rel cysteine mutants. Black bars represent the absence of DTT, whereas gray bars represent its presence.

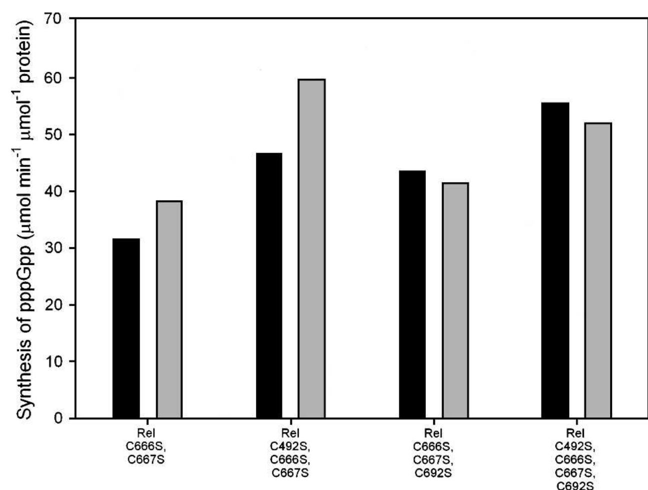

Figure 11. Effect of 40 μg uncharged tRNA on the synthesis activity of Rel cysteine mutants. Black bars represent the absence of tRNA, whereas gray bars represent its presence.

tRNA, unlike RelA from *Streptomyces coelicolor* (Martinez-Costa et al. 1998) and *E. coli* (Haseltine and Block 1973). Similar findings have been reported in the case of Rel_{Mtb} (Avarbock et al. 1999). However, in the presence of uncharged tRNA, the pppGpp synthesis activity of Rel WT increases significantly as compared with Rel NTD, which does not show such a phenomenon. Similar observations were made in a study on Rel_{Mtb}, wherein tRNA positively affected the synthesis activity of full-length Rel protein and did not show such effect in the C-terminal-deleted version (Avarbock et al. 2000, 2005). This therefore clearly demonstrates that tRNA binds to the C-terminal part of the protein and thus modulates the activity of Rel WT protein. One should keep in mind here that the net pppGpp synthetic activity of Rel NTD is always more than the native Rel. Although we found a slight reduction in the synthesis activity of Rel NTD in the presence of tRNA, the change was insignificant, and therefore this point was not pursued any further. The deleted constructs were not only active under in vitro conditions but were active in vivo as well, as revealed by the synthesis of (p)ppGpp in Δ*rel* *M. smegmatis* cells. There could be other mechanism(s) as well to activate Rel protein in vivo in the case of *M. smegmatis*.

We report here that Rel_{Msm} protein forms multimers after purification, though the protein is monomer in vivo. As there lies a distinct possibility that the Rel monomer-to-multimer equilibrium in vivo may have a regulatory role and could not be detected by the Western blotting used here, we decided to investigate the role of cysteine residues in Rel multimerization as well as in biological activity. The conservation of these residues among different organisms also prompted us to proceed further. Of six cysteines present in this protein (C332, C338, C492, C666, C667, C692), four present in the C-terminal half are involved in multimerization. We hypothesize that the cysteines present at the C-terminal half of the protein are exposed to the solvent and therefore are easily accessible for the formation of multimers. As the activity of the Rel NTD fragment was independent of DTT or tRNA concentration, we inferred that the C-terminal is involved in regulation. The proteins were run in the presence of 6 M urea with or without 10 mM DTT on a Superdex 200 10/300 GL gel-filtration column to further confirm the multimerization status of the proteins.

The cysteines in Rel_{Msm} were mutated to serines in order to obtain a monomeric protein. We found that the protein forms dimers with different mobilities in the case of two different cysteine triple mutants. Rel_{Msm} was found to be monomer upon conversion of all four C-terminal cysteines to serines. The synthesis activity of these proteins increased as a function of cysteine mutation, which further proved our finding that the monomer is the functional unit.

Mutation of C692 to serine showed an interesting property. This cysteine was found to be conserved among all of the Rel proteins from mycobacterial species. Moreover, it was also conserved in the *E. coli* RelA. We found that the Rel_{Msm} C692S protein did not show any increase in the synthesis activity upon addition of uncharged tRNA. We thus conclude here that the C692 is required for interaction with uncharged tRNA, and mutation of cysteine to even a very closely related amino acid-like serine makes the protein nonresponsive to uncharged tRNA. This result is also supported by an earlier observation in *E. coli* RelA that mutation of C638, which aligns with C692 in Rel_{Msm} (Fig. 1C), renders the protein inactive in its regulatory function (Gropp et al. 2001).

Our CD experiments with Rel WT and Rel NTD proteins indicated that Rel WT protein is mostly α-helical in nature as noted from the estimation of the percent secondary structure. The most notable observation was that Rel NTD, where the C-terminal half was deleted, showed similar far-UV CD profile to the Rel WT. The calculated percent secondary structure content of Rel NTD was found to be close to that of the Rel_{Seq} crystal structure data (PDB ID 1VJ7; Hogg et al. 2004). Based on these observations and also on the fact that the C-terminal half is involved in multimerization, we hypothesized that the C terminus of the Rel WT protein should be, at least partially, unstructured, and therefore will be masked by the strong CD signal of the Rel WT protein. It appears that the fusion of a structured N-terminal domain of Rel with unstructured C-terminal domain alters the overall conformation of the protein. Keeping these points in mind, we went ahead with the cloning, expression, and purification of Rel CTD (only TGS and ACT domains present). The protein did not have any synthesis or

hydrolysis activity, thus confirming that the catalytic activity is confined to the N-terminal half of the protein. Interestingly, the CD profile of the C-terminal domain, i.e., Rel CTD, showed reduced secondary structure content, if any, as noted from the calculated percent secondary structure. Moreover, the urea denaturation profile of Rel CTD is suggestive of a noncooperative unfolding, and, at the moment, we believe that the protein is largely unstructured. We do not know the significance of this protein being unstructured, as it does not play any role in multimerization in vivo. Since Rel CTD bears TGS and ACT domains, both of which are involved in regulating the two activities of the Rel WT protein, we are tempted to postulate that the Rel CTD protein will attain structure upon binding to ligand (amino acids, nucleotides, or uncharged tRNA) and this process will lead to the activation of Rel protein.

Materials and methods

Bacterial strains, plasmids, and growth conditions

Plasmids and oligonucleotides used in this study are listed in Table 3. *E. coli* strains DH5α and BL21 (DE3) were grown in LB broth at 37°C with agitation or on a plate containing 1.5% agar. Ampicillin (100 μg/mL) or Kanamycin (50 μg/mL) was used as and when required. *M. smegmatis* strain mc^{2}155 was grown in MB7H9 (Difco) broth or on a plate containing 1.5% agar. Kanamycin (30 μg/mL) or Hygromycin (30 μg/mL) was used as and when required. All of the PCR reactions were carried out using Dynazyme EXT polymerase (Finnzyme) with the manufacturer's instructions. All of the clones generated were confirmed by sequencing (Microsynth). Restriction enzymes used for the cloning were procured from New England Biolabs.

Conserved Domain Database search of Rel protein

The Rel protein sequence was submitted to NCBI Conserved Domain Database search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) to identify various domains present in this protein. All of the default parameters suggested at the Web site were used for this analysis. The databases used were CDD v2.03 and Oasis_Pfam.v2.03.

Sequence alignment of Rel protein from different organisms

Rel protein sequences from *M. tuberculosis*, *M. smegmatis*, *M. leprae*, and *E. coli* were submitted for multiple sequence alignment in ClustalW (at http://www.ebi.ac.uk/clustalw) using default parameters. Comparison of any two sequences was made by pairwise alignment using Emboss align (at http://www.ebi.ac.uk/emboss/align) using standard parameters.

Cloning, expression, and purification of Rel WT protein

Rel protein-coding gene (2394 bp, 797 amino acids) was PCR amplified from *M. smegmatis* genomic DNA using a set of primers, Suj1 and Suj2 (Table 3). The amplicon was digested with NdeI and NotI and was cloned in pET21b plasmid predigested with the same enzymes. The resulting plasmid pETRelWT was transformed in *E. coli* BL21 (DE3) for protein expression and purification. Cells were grown in LB medium until OD ~0.6, induced with IPTG to a final concentration of

Table 3. *Different strains and plasmids used in this study*

Plasmids	Relevant genotype	Source or reference
pMOSBlue	Vector predigested with EcoRV for blunt end ligation, Amp^r	Amersham Biosciences
pET21b	Expression vector with hexa-histidine tag getting expressed at the C terminus of the protein, Amp^r	Novagen
pETRelWT	Rel WT protein gene *rel* cloned in pET21b at NdeI and NotI sites	This study
pETRelACT	C terminus ACT domain deleted *rel* cloned in pET21b at NdeI and HindIII sites	This study
pETRelNTD	C terminus ACT and TGS domains deleted *rel* cloned in pET21b at NdeI and HindIII sites	This study
pETRelCTD	C terminus ACT and TGS domains cloned in pET21b at NheI and NotI sites	This study
pETRelRSD	RSD domain of Rel WT protein cloned in pET21b at NheI and HindIII	This study
pMV261	A *Mycobacterium–E. coli* shuttle vector, Kan^r, hsp60 promoter	Stover et al. 1991
pMVRelWT	Rel WT protein gene *rel* cloned at PvuII and EcoRI sites in pMV261	This study
pMVRelACT	C terminus ACT domain deleted *rel* cloned at PvuII and EcoRI sites in pMV261	This study
pMVRelNTD	C terminus ACT and TGS domains deleted *rel* cloned at PvuII and EcoRI sites in pMV261	This study
	Oligonucleotides	
Suj1	5′-GGAGGTGACACATATGGTCGACGAGCCAGGC	
Suj2	5′-GCCGAACCGCTCGATGCGGCCGCGCTGGTG	
RELACT	5′ CGAGTGCCTCAAGCTTGATGGCGACC	
RELTGS	5′-GTGAACACGAAAAGCTTCTGCGTGG	
RELCO_F	5′-AAGAAGGAGATAGATATCTGGTCGACG	
RELCO_R	5′-GCAGCAGCGAATTCAGCTTCCTTTCG	
Relctfor	5′-CTCGCCACGCAGGAGATCTTCGTGTTCACG	
Nterrev	5′-TCAGTGGTGGTGGTGGTGGTGCTCGAGTG	
RSDOMFOR	5′-GTGGCGGCTAGCGCCATGAAGATCAACG	

1 mM, and further grown for 3 h. Cells were harvested by centrifugation at 4000 rpm for 10 min and lysed in lysis buffer (50 mM Tris-Cl at pH 7.9, 500 mM NaCl, 1 mM PMSF) by two rounds of sonication of 5 min each with a 5-sec pulse at 4°C, and the lysate cleared by centrifugation at 15,000 rpm for 15 min at 4°C. The supernatant was loaded on Ni-NTA column pre-equilibrated with the equilibration buffer containing 50 mM Tris-Cl (pH 7.9) and 500 mM NaCl. The column was washed with 100 column volumes of wash buffer containing 50 mM Tris-Cl (pH 7.9), 500 mM NaCl, and 10 mM imidazole, and the protein was eluted with the elution buffer (50 mM Tris-Cl at pH 7.9, 500 mM NaCl, and 500 mM imidazole). Eluted protein was then dialyzed against a buffer containing 50 mM Tris-Cl (pH 7.9), 500 mM NaCl, and stored at 4°C for future use.

Cloning, expression, and purification of Rel dACT and Rel NTD

For the construction of Rel dACT (amino acids 1–722) and Rel NTD (amino acids 1–457), pETRelWT was used as a template, and using a set of primers, Suj1 and RELACT (for Rel dACT) or RELTGS (for Rel NTD) (Table 3), the required region was PCR amplified and cloned in pET21b vector at NdeI and HindIII sites, thus resulting in either pETReldACT or pETRelNTD. For expression and purification of the protein, strategy similar to that of Rel WT was followed.

Cloning, expression, and purification of RelA_SpoT domain of Rel_{Msm}

The RelA_SpoT domain (RSD domain, fragment 290–457) of Rel protein was cloned separately in order to obtain the smallest segment that can synthesize (p)ppGpp in vitro and/or in vivo. The RSD domain region was PCR amplified using a set of primers, RSDOMFOR and Nterrev (Table 3) and pETRelNTD as template. The PCR product was digested with NheI and HindIII and was subcloned into pET21b predigested with the same enzymes, thus giving rise to pETRelRSD. This construct was used to transform *E. coli* BL21 (DE3) for protein preparation. It was observed that even after growing the cells at 12°C and inducing it at an OD_{600} ~0.6 with 0.05 mM IPTG concentration, the expressed protein only enriched the inclusion body. Therefore, the protein was purified from the inclusion bodies by growing the cells at 37°C until the OD_{600} ~0.6, after which it was induced by an addition of 0.5 mM IPTG. The cells were then harvested by centrifugation at 12,000 rpm for 10 min at 4°C and lysed by incubation in lysis buffer containing 50 mM Tris-Cl (pH 7.9), 500 mM NaCl, and 6 M Urea at 4°C for 3–6 h. Sonication was avoided. The lysate was cleared by centrifugation at 15,000 rpm for 15 min at room temperature, and the supernatant was loaded on Ni-NTA column pre-equilibrated with the equilibration buffer containing 50 mM Tris-Cl, 500 mM NaCl, and 6 M Urea. The column was washed with 100 column volumes of wash buffer containing 50 mM Tris-Cl (pH 7.9), 500 mM NaCl, 6 M Urea, and 10 mM imidazole. Protein was then eluted with the elution buffer (50 mM Tris-Cl at pH 7.9, 500 mM NaCl, 6 M Urea, and 500 mM imidazole) and dialyzed against a refolding buffer containing 50 mM Tris-Cl (pH 7.9), 500 mM NaCl, and stored at 4°C. Concentration of the protein was measured after refolding by recording the A_{280} and calculating it using its absorption coefficient value as given in Table 4 keeping refolding buffer as blank.

Cloning, expression, and purification of Rel CTD

To construct pETRelCTD (amino acids 453–797), the required fragment was PCR amplified using a set of primers, Relctfor and Nterrev (Table 3) and pETRelWT as template and was cloned in the pMOSBlue (Amersham Biosciences) using blunt-end ligation as per the manufacturer's instructions. The fragment was subsequently released by using NdeI and NotI enzymes and was then subcloned in pET21b vector predigested with the same enzymes. Protein was expressed and purified using conditions employed for Rel WT.

Cloning of full-length and deleted versions of Rel protein in Mycobacterium–E. coli *shuttle vector pMV261*

The full-length and deleted versions of Rel protein were PCR amplified from their pET21b counterparts using primers RELCO_F and RELCO_R (Table 3), digested with EcoRV and EcoRI, and ligated in pMV261 (a mycobacterial–*E. coli* shuttle vector [Stover et al. 1991]) predigested with PvuII and EcoRI enzymes. The genes were cloned along with a His-tag at the C terminus. After clone selection, the vectors pMVRelWT (pMV261 vector containing full-length *rel*), pMVReldACT (Rel dACT construct), and pMVRelNTD (Rel NTD construct) were transformed in the *M. smegmatis* mc^2155 Δ*rel* strain (genomic copy of *rel* replaced with hyg^r cassette [Mathew et al. 2004]).

Cysteine mutagenesis

Of the six cysteines present in the Rel WT protein, four were mutated using the protocol as described (Shenoy and Visweswariah 2003) with slight modification. A "two-step mutagenesis" strategy was followed wherein in one step a restriction site was introduced, and in the second step it was removed and the original amino acid was changed to serine. This process also aided in the screening of mutants. All of the mutations were finally confirmed by sequencing at Microsynth.

Mass spectrometric analysis

All purified proteins were subjected to tryptic digestion, and peptide mass fingerprints were recorded on a Bruker UltraFlex MALDI TOF mass spectrometer; their identities were confirmed using MASCOT database analysis (Perkins et al. 1999). Molecular weights of the full-length protein as well as the mutants were also recorded.

Table 4. *Molecular masses and absorption coefficients of different proteins used in this study*

Protein (with hexa-histidine tag)	Molecular mass (kDa)	Absorption coefficient at A_{280} (M^{-1} cm^{-1})	A_{280} = 1 mg/mL
Rel WT	~89	76810	0.862
Rel dACT	~81	74250	0.915
Rel NTD	~52	53340	1.017
Rel CTD	~39	23470	0.605
RSD domain	~21	39970	1.913

In vitro pppGpp synthesis assay

The pppGpp synthesis activity was assayed in a 5-μL reaction mixture containing 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 100 mM NaCl, 4 mM ATP (1 μCi/μmol), 13 mM GTP, and 5 μM of desired proteins (Rel WT/Rel dACT/Rel NTD/Rel RSD/Rel CTD). [γ^{32}P]ATP was obtained from Perkin-Elmer (specific activity 6000 Ci/mmol). The protein concentration was measured by checking the absorbance at 280 nm and calculating it with the extinction coefficient of the same at 280 nm. The values for the extinction coefficient for different proteins used, obtained by submitting the Rel WT and deleted versions' protein sequences at http://tw.expasy.org/tools/protparam.html, are given in Table 4. The reaction mixture was incubated at 37°C for 30 min, after which it was arrested by adding 1 μL of 6 M formic acid. After centrifugation at 13,000 rpm at 4°C for 10 min, 5 μL of the supernatant was spotted on a PEI-coated TLC sheet (Merck). It was then developed in 1.5 M KH_2PO_4 (pH 3.4) in ascending chromatography, air-dried, and PhosphorImaged (Fujifilm FLA2000). Reaction mixture lacking any protein was used as negative control. Spots obtained were analyzed by densitometry. The effect of dithiothreitol on the activity of Rel WT, the cysteine mutants in full-length Rel, and Rel NTD proteins was monitored using 10 mM concentration of DTT. The effect of uncharged tRNA was observed using 40 μg total concentration of *E. coli* tRNA Type XXI (Sigma) added to a similar reaction mixture as described above containing 5 μM of any of the proteins.

pppGpp synthesis activity was also monitored in the presence of both Rel NTD and Rel CTD by in vitro complementation. The activity assay was done with 5 μM of Rel NTD with different concentrations of Rel CTD in the assay conditions as mentioned. Reaction mixture in the absence of any protein was used as negative control.

All of the assays were repeated at least thrice, and the data presented here are the average. The errors were in the limit of 3% in all the cases.

Measurement of the (p)ppGpp synthesis in vivo

To measure the in vivo synthesis of (p)ppGpp, the full-length and deleted versions of Rel protein were cloned in mycobacterial–*E. coli* shuttle vector pMV261 (Stover et al. 1991) downstream to *hsp60* promoter and the resulting constructs were transformed in *M. smegmatis* Δ*rel* strain (Mathew et al. 2004). The cells harboring these plasmids were grown in 3 mL MB7H9 broth containing 30 μg/mL kanamycin at 37°C. At an OD ~0.2, the cells were labeled by adding $H_3{}^{32}PO_4$ (>3000 Ci/mmol, BRIT, Hyderabad, India) (final concentration of 100 μCi/mL) directly to the culture medium. Cells were harvested after 12 h, washed once with 10 mM Tris-HCl (pH 8.0), resuspended in 50 μL of the buffer, treated with 1 mg/mL of lysozyme on ice for 20 min, and lysed by incubating with 1% SDS for 3 h. (p)ppGpp was extracted with an equal volume of 2 M formic acid. After centrifugation at 13,000 rpm at 4°C for 10 min, 5 μL of the supernatant was spotted on a PEI-coated TLC plate (Merck), developed, and imaged as described above. *M. smegmatis* Δ*rel* strain harboring wild-type pMV261 plasmid was used as a negative control. Mobility of the extracted (p)ppGpp was confirmed by running the standard alongside.

Large-scale synthesis of pppGpp

pppGpp was synthesized at a large scale as already described (Krohn and Wagner 1995). Briefly, in a 2-mL reaction volume, 6 mM ATP and 3 mM GTP (1 μCi/μmol γ^{32}P-GTP) were incubated in 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and 200 μg/mL of Rel NTD at 37°C for 5 h, after which the reaction was stopped with the addition of formic acid to a final concentration of 1 M. The reaction mixture was then filtered through a 0.22-μM filter (Sartorius) and injected in Protein PAK G-DEAE anion exchange column (Waters) pre-equilibrated with 50 mM Tris (pH 7.5) at a flow rate of 0.5 mL/min. pppGpp was eluted with a 0.0–0.5 M linear gradient of NaCl. The peak fractions containing pppGpp were pooled and lyophilized and subjected to a Sephadex G-10 (Sigma) gel filtration. The peak fractions were again pooled and lyophilized, and the specific activity was adjusted to 0.1 μCi/μmol. The concentration of pppGpp was calculated by measuring its absorbance at 252 nm with its extinction coefficient at $A_{252} = 13{,}100\ M^{-1}cm^{-1}$ (Krohn and Wagner 1995).

pppGpp hydrolysis assay

Hydrolysis assay was performed with all the Rel constructs as described (Mechold et al. 1996) in the presence or absence of 10 mM DTT. Briefly, 5 μM of protein was incubated at 37°C in a reaction mixture containing 2 mM pppGpp (0.1 μCi/μmol), 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM DTT, and 0.1 mM EDTA. Final concentration of NaCl in the reaction mixture was not allowed to exceed 100 mM. The reaction was incubated for 30 min and stopped by adding formic acid to a final concentration of 1 M. The mixture was centrifuged at high speeds briefly, and 5 μL of the supernatant was spotted on a PEI-coated cellulose TLC sheet, which was then developed in 1.5 M KH_2PO_4 (pH 3.4), dried, and PhosphorImaged. The densitometric analysis was performed on the product GTP or the remaining amount of pppGpp. Reaction lacking protein was used as negative control.

Rel multimerization studies

To look for the oligomeric species, Rel WT, Rel dACT, Rel NTD, and Rel CTD proteins were examined on a denaturing polyacrylamide gel (Laemmli 1970) with a modified loading buffer (50 mM Tris-Cl at pH 6.8, 0.1% bromophenol blue and 10% glycerol). Samples were incubated in the presence or the absence of DTT (final concentration 100 mM) and/or SDS (final concentration 2.0%). Either SDS or DTT, or both were used to see whether the formed oligomers were disulfide-bonded. The samples were boiled in the presence of SDS and in the absence of DTT to see whether any noncovalent interactions were responsible for multimerization. In another experiment, the protein was loaded on an 8% denaturing polyacrylamide gel (first dimension) in the absence of DTT in more than one lane and was run parallel. A gel strip was cut out from one of the lanes after completion, incubated with 50 mM Tris-Cl (pH 6.8), 100 mM DTT, and 0.1% bromophenol blue for 5 min, and then loaded on another 8% denaturing polyacrylamide gel (second dimension). The gel was stained with Coomassie Blue R 250.

Circular dichroism studies of the Rel WT, Rel NTD, and Rel CTD proteins

CD spectra were recorded at 22°C on a Jasco J-715 spectropolarimeter. Proteins were dialyzed against 10 mM phosphate buffer (pH 8.0) containing 150 mM KCl. A total of 2 μM of Rel

WT, 3 μM of Rel NTD, or 3 μM of Rel CTD was used for CD studies. Protein samples were filtered through a 0.2-μm filter (Sartorius) prior to use. Scans were taken using bandwidth 2.0 nm, response time 2 sec, data pitch 0.2 nm, and scanning speed of 50 nm/min from 250 nm to 200 nm in a quartz cuvette of path length 2 mm. Spectra were averaged over five scans and buffer-subtracted. For studying urea denaturation of proteins, both Rel NTD and Rel CTD (3 μM each) were incubated with different concentrations of urea ranging from 0.0 to 6.0 M for 5 h and filtered through a 0.2-μm filter (Sartorius) prior to use. CD at 222 nm was recorded using conditions as described above. At least five scans were accumulated, averaged, and buffer-subtracted in each case.

The secondary structure estimation (%helix, %sheet, and %unordered) was carried out using the programs CDSSTR (Compton and Johnson 1986; Sreerama and Woody 2000) and CONTINLL (Provencher and Glockner 1981; van Stokkum et al. 1990) as obtained from http://lamar.colostate.edu/~sreeram/CDPro/index.shtml. The instructions provided for using the programs were followed. The wavelength range used was 200–240 nm. At least, two different protein data sets viz. 43 soluble proteins (SP43) and 43 soluble plus five denatured proteins (SDP48), as provided in the software, were used for calculation and compared.

Western analysis

Western analysis was performed with Rel WT protein in order to find out the in vivo status of Cys residues in *E. coli*. The method described by Kishigami et al. (1995) was followed. Briefly, *E. coli* BL21 (DE3) harboring pETRelWT was grown until an OD_{600} ~0.6. The culture was then induced with IPTG to a final concentration of 1 mM, after which the cells were further grown for 3 h. The culture was mixed with an equal volume of 10% TCA to lyse the cells and precipitate the whole-cell protein. The precipitate was recovered by centrifugation at 12,000 rpm for 5 min, washed once with ice-cold acetone, and then resuspended in 50 mM Tris-Cl (pH 8.0), 1 mM EDTA, 1% SDS containing 50 mM iodoacetamide. The protein was then separated on an 8% denaturing polyacrylamide gel without adding any reducing agent. The protein was visualized by immunoblotting with anti-Rel antibodies raised in rabbit here or with anti-His-tag antibodies (Sigma).

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