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Nucleoside diphosphate kinase of *Mycobacterium tuberculosis* acts as GTPase-activating protein for Rho-GTPases

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Abstract Several bacterial pathogens secrete proteins into the host cells that act as GTPase-activating proteins (GAPs) for Rho-GTPases and convert GTP-bound active form to GDP-bound inactive form. However, no such effector molecule has been identified in *Mycobacterium tuberculosis*. In this study, we show that culture supernatant of *M. tuberculosis* H_{37}Rv harbors a protein that stimulates the conversion of GTP-bound Rho-GTPases to the GDP-bound form. Nucleoside diphosphate kinase (Ndk) was identified as this culture supernatant protein that stimulated in vitro GTP hydrolysis by members of Rho-GTPases. The histidine-117 mutant of Ndk, which is impaired for autophosphorylation and nucleotide-binding activities, shows GAP activity. These results suggest that Ndk of *M. tuberculosis* functions as a Rho-GAP to downregulate Rho-GTPases, and this activity may aid in pathogenesis of the bacteria.

Keywords: Nucleoside diphosphate kinase; Tuberculosis; G-protein; GTPase activating protein; Actin cytoskeleton; *Mycobacterium*

1. Introduction

Rho-GTPases perform a complex array of functions through various downstream proteins. The activity of these proteins are under the tight regulation of multiple regulatory proteins, including GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs), responsible for deactivation and activation of these proteins, respectively [1]. Several pathogenic bacteria secrete proteins that act as either GAP or GEF, and eventually facilitate their pathogenesis [2]. Intracellular pathogen, *Salmonella typhimurium* has been shown to secrete a protein, SptP, which acts as GAP for members of Rho-GTPases and helps in the host-cell recovery after bacterial invasion [3]. Secreted cytotoxin, ExoS of *Pseudomonas aeruginosa*, disrupts the actin cytoskeleton by acting as GAP for Rho-GTPases [4]. Similarly, *Yersinia pseudotuberculosis* secretes a cytotoxic factor, YopE, which depolymerizes the actin stress fiber, through its GAP activity for Rho-GTPases [5]. The mechanism of entry of *M. tuberculosis* inside the host cell is still not clear. However, *M. avium* has been shown to gain entry into the intestinal epithelium cells by disrupting the macrophage actin filament network by activating Rho proteins [6]. Role of Rho-GTPases in the pathogenesis of *M. tuberculosis* has not yet been studied.

Nucleoside diphosphate kinase (Ndk) has been found to be a multifunctional enzyme, as besides a central role in nucleotide metabolism, it has been implicated in a variety of biochemical and cellular regulatory processes [7]. Recently, we reported that *M. tuberculosis* secretes Ndk, which is cytotoxic for macrophages [8]. Moreover, Ndk from various organisms have been shown to interact with many G-proteins such as trimeric G-proteins [9–11], members of Ras family [12], ADP ribosylation factor [13], tubulin [14] and with small GTP-binding proteins [15]. In this study, for the first time we report that culture supernatant (C. sup) of *M. tuberculosis* harbors a Rho-GTPases inactivating protein. This activity was found to be associated with Ndk. These results suggest that Ndk of *M. tuberculosis* acts as GAP for members of Rho-GTPases. Thus, Ndk of *M. tuberculosis*, which plays a crucial role in nucleotide metabolism, may also aid in the pathogenesis of *M. tuberculosis*.

2. Materials and methods

2.1. Expression and purification of proteins

Rho-GTPases (RhoA, RacI and Cdc42) were purified as N-terminal GST-tagged proteins. Cloning of genes encoding for members of Rho-GTPases; RhoA, RacI and Cdc42, expression in *Escherichia coli*, purification, and biochemical characterization of the recombinant proteins (GST-tagged) will be described elsewhere (Chopra et al., in preparation). Ndk and its mutant protein (H117Q) were expressed in *E. coli* and purified as histidine-tagged proteins using Ni–NTA resin as described earlier [8].

2.2. Preparation of culture supernatant

Mycobacterium tuberculosis H_{37}Rv was cultured in Middlebrook 7H9 medium supplemented with 10% ADC and 0.2% Tween 80 at 37 °C with shaking (220 rpm) for 3–4 weeks. The mid log phase C. sup was filtered through a 0.22 μm filter and concentrated 50-fold using centricon-10 concentrators.

2.3. GTP hydrolysis assays

GTP hydrolysis activity was determined by filter binding assay as described earlier with minor modifications [16]. In brief, Rho-GTPases

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(1 μg) were incubated separately with 10 μCi of either [α-^{32}P]GTP or [γ-^{32}P]GTP in 100 μl of a solution containing 50 mM HEPES (pH 7.4), 50 mM NaCl, 0.1 mM DTT, 5 mM EDTA, and 1 mg/ml BSA, for 10 min at 37 °C. After incubation, 10 mM of $MgCl_2$ was added and the reaction was incubated in ice for 10 min. Samples were spotted on nitrocellulose (NC) filters, washed extensively with cold assay buffer to remove any unbound radioactivity and air-dried. Concentrated C. sup of *M. tuberculosis* was added on the NC membrane and incubated at 37 °C for 1 h. After incubation, the NC membranes were washed twice with cold buffer and air-dried. Filter-associated radioactivity was determined by autoradiography and by liquid scintillation counting (Beckman, USA).

Effects of boiling the C. sup (10 μg) on its GAP activity was examined by boiling the C. sup for 10 min, prior to incubation with GTP loaded Rho-GTPases. The effect of phosphatase and protease inhibitors on the C. sup's GAP activity was examined by carrying the reaction by pre-incubating the C. sup with phosphatase inhibitor (sodium fluoride) and protease inhibitor (aprotinin) in separate reaction mixtures. Similarly, the effect of anti-Ndk antibodies was examined by pre-incubating the C. sup with anti-Ndk antibodies (1:500 dilution) for 20 min prior to addition of GTP loaded Rho-GTPases.

2.4. GAP assays with purified Ndk and its mutant protein

Two methods were used to examine the GAP activity of purified Ndk for the Rho-GTPase family of proteins (RhoA, RacI, and Cdc42) [17].

In the first method, GTP hydrolysis was assayed by pre-loading GSH–Sepharose beads bound GST-Rho-GTPases (1 μg) with [α-^{32}P]GTP (3 μCi) in buffer A (50 mM Tris–HCl [pH 7.4], 1 mM $MgCl_2$, 1 mM DTT, and 1 mg/ml BSA) at 25 °C for 5 min. After completion of incubation period, Sepharose beads were washed thrice with buffer A at 4 °C to remove unbound radioactivity. The Rho-GTPase loaded with [α-^{32}P]GTP was incubated with or without Ndk (1 μg) at 25 °C for 5 min. At the end of incubation period, Sepharose beads were washed twice with buffer A and bound nucleotides were eluted with 20 μl of elution buffer (1% SDS and 50 mM EDTA) at 65 °C for 5 min. The labeled nucleotides were resolved on polyethyleneimine-thin layer chromatography (PEI-TLC) plates with a mobile phase of 0.75 M KH_2PO_4 (pH 3.4) and finally developed by autoradiography.

In the second method, time-dependent GAP activity of Ndk was examined by incubating Ndk with GSH–Sepharose beads bound GSt-Rho proteins, that were pre-loaded with 10 μCi of [γ-^{32}P]GTP. Procedure was the same as described above. Nucleotides were eluted using elution buffer and their remnant radioactivity measured using scintillation counter. Decrease in counts in the presence of Ndk is a measure of GAP activity of Ndk.

3. Results and discussion

3.1. Culture supernatant of M. tuberculosis *harbors Rho-GTPases inactivating protein*

Mycobacterium tuberculosis sustains a prolonged association with its host by interfering with macrophage signaling processes. Small GTP-binding proteins are known to play a crucial role in the signal transduction of phagocytosis [18]. One approach to understand this mechanism would be to determine the presence of proteins in *M. tuberculosis*, which might interfere with host cell signaling.

In an attempt to explore the possibility that *M. tuberculosis* being an intracellular pathogen might secrete Rho-GTPases modulating proteins, C. sup of *M. tuberculosis* was examined for the presence of such proteins. C. sup stimulated the GTP-hydrolyzing activity of Rho-GTPases in a time-dependent manner with about 50% hydrolysis of bound GTP in 10 min (data not shown), and complete hydrolysis was observed after 1 h of incubation (Fig. 1A). Boiling of C. sup resulted in complete loss of activity, suggesting the association of this activity with a protein (Fig. 1B and E). The above results obtained with the C. sup might be due to one of these activities: (a) a protein that can promote nucleotide dissociation from Rho-GTPases; (b) a protease that can degrade Rho-GTPases; (c) a phosphatase that removes phosphate from

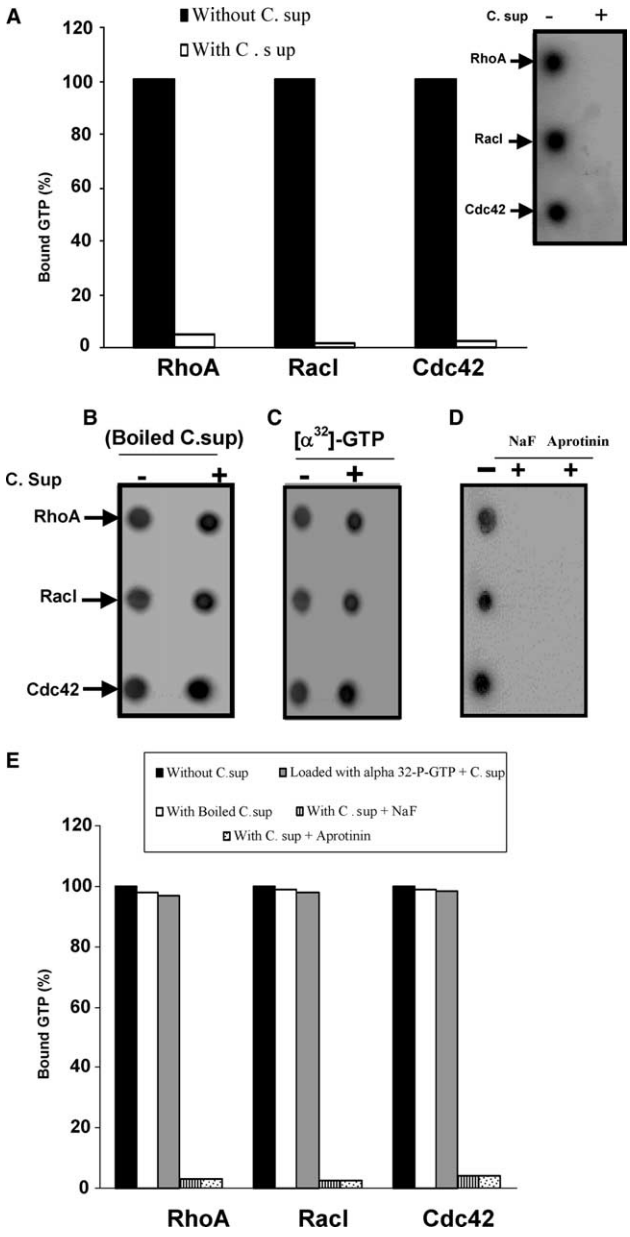

Fig. 1. GAP activity of Culture supernatant of *M. tuberculosis*. GSt-Rho/Rac/Cdc42 proteins were loaded with 10 μCi of [γ-^{32}P]GTP at 37 °C for 10 min, spotted on nitrocellulose membrane and washed thrice. The [γ-^{32}P]GTP loaded Rho-GTPases were incubated with or without C. sup of *M. tuberculosis* at 37 °C for 1 h in separate reaction mixtures. At the end of incubation period, membranes were washed twice, dried and developed by autoradiography. Decrease in counts in the presence of C. sup is a measure of GAP activity. (A) [γ-^{32}P]GTP loaded RhoA, RacI and Cdc42 were incubated with or without C. sup of *M. tuberculosis* $H_{37}Rv$ for 1 h and filter-associated radioactivity was quantified by liquid scintillation counter. Inset shows corresponding TLC; (B) C. sup was boiled and incubated with [γ-^{32}P]GTP loaded Rho-GTPases; (C) C. sup was incubated with [α-^{32}P]GTP loaded Rho-GTPases; (D) C. sup was pre-incubated with NaF and aprotinin for 20 min before incubation with [γ-^{32}P]GTP loaded Rho-GTPases; and (E) corresponding filter-associated radioactivity of B–D was quantified by liquid scintillation counter.

Rho-bound GTP; or (d) a protein that specifically promotes the release of [γ-^{32}P]phosphate from Rho-GTP. Attempts were made to evaluate these possibilities. The first possibility was examined by carrying out the reaction of C. sup with Rho-GTPases loaded with [α-^{32}P]GTP. We observed that, unlike γ-phosphate-labeled GTP, Rho-bound α-phosphate was retained on the filter after incubation with C. sup of *M. tuberculosis* (Fig. 1C and E). These results indicate that the observed activity is due to the selective loss of γ-phosphate from Rho-GTPases, and is not because of degradation of Rho protein or effect of GTPases present in the culture supernatant on the released nucleotides. To address the second and third possibilities, we performed the assay in the presence of aprotinin and sodium fluoride. These inhibitors failed to inhibit the GTPase-stimulating activity of the C. sup, implying that this activity is not due to phosphatases or proteases present in the C. sup (Fig. 1D and E). Collectively, these results suggest that the mycobacterial C. sup harbors a protein with GAP activity.

3.2. GTPase inactivating protein in the culture supernatant of M. tuberculosis *H37Rv is Ndk*

Mycobacterium tuberculosis is known to secrete a large number of proteins into the culture medium and these proteins play crucial roles in the mycobacterial–host cell interaction. In the last few years, there are several reports suggesting that Ndk from various organisms act as G-proteins regulators [9–17]. Human homologue of Ndk, Nm23H1 is shown to be a bi-functional regulator (acting as both GAP and GEF) of Rad, a prototype of a Ras-related GTPase subfamily [17]. In a recent study, Ndk of *E. coli* has been shown to inactivate oncogenic Ras-GTPases [16]. However, to date there is no report of interaction of Ndk from any organisms with members of Rho-GTPases.

The above observations, and secretory nature of Ndk of *M. tuberculosis* prompted us to speculate that Ndk might be a possible regulator of Rho-GTPases. In an earlier study, polyclonal antibodies raised against Ndk of *Dictyostelium* acted as strong inhibitors of its enzymatic activity [19]. Interestingly, pre-incubation of C. sup of *M. tuberculosis* with anti-Ndk antibodies, prior to the addition of Rho-GTPases pre-loaded with [γ-^{32}P]GTP, resulted in about 70% reduction of C. sup associated GAP activity (Fig. 2), suggesting that GAP activity was mainly attributed to Ndk present in the C. sup. Complete loss in activity was not observed because some other C. sup proteins might also have GAP activity towards Rho-GTPases.

3.3. Ndk acts as GTPase activating protein for Rho family of GTPases

In this study, an effort was made to evaluate the ability of Ndk to stimulate the intrinsic GTPase activity of members of Rho-GTPases. GAP activity of Ndk was examined by two methods. In the first method, Ndk was incubated with GSH–Sepharose resin bound-[γ-^{32}P]GTP loaded Rho-GTPases (RhoA, RacI and Cdc42). As shown in Fig. 3, Ndk increased the intrinsic rate of GTP hydrolysis of the members of Rho-GTPases in a time-dependent manner. It was observed that incubation of Ndk with [γ-^{32}P]GTP loaded Rho-GTPases resulted in about 72% bound GTP hydrolysis in a minute. Whereas, the half time for the intrinsic rate of GTP hydrolysis by Rho-GTPase was about 18 min. Similarly, incubation of Ndk with [γ-^{32}P]GTP loaded RacI and Cdc42 resulted in about 79% and 74% hydrolysis of GTP, respectively, after 1 min, whereas, half-life for the intrinsic GTPase activity of RacI and Cdc42 were 18 and 12 min, respectively. Heat inactivated (100 °C for 10 min) Ndk failed to show GAP activity (data not shown). In the second method, GAP activity of Ndk was examined by incubating GSH–Sepharose beads bound GST-Rho/Rac/Cdc42 proteins pre-loaded with [α-^{32}P]GTP. Ndk enhanced the Rho-GTPases mediated conversion of [α-^{32}P]GTP to [α-^{32}P]GDP (Fig. 4A–D).

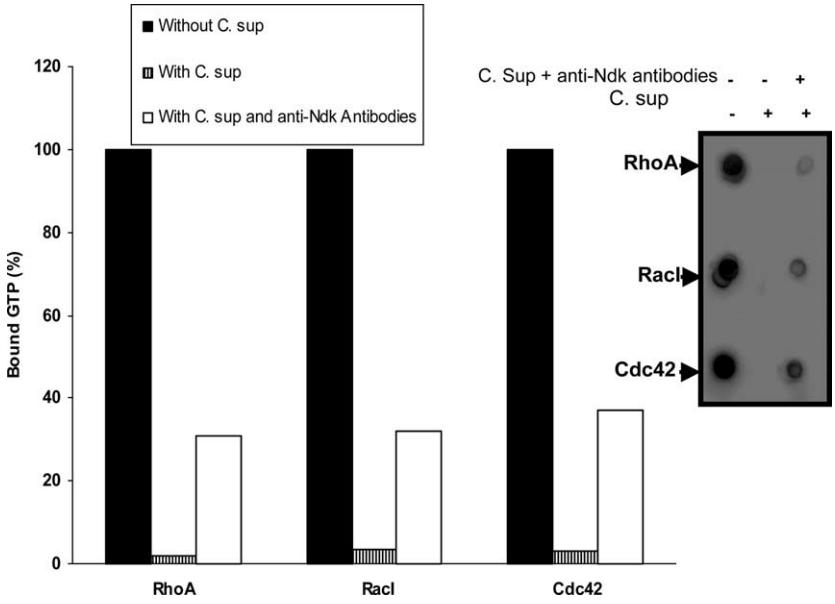

Fig. 2. GTPase inactivating protein in the culture supernatant is Ndk. [γ-^{32}P]GTP loaded RhoA, RacI and Cdc42 were incubated with or without C. sup of *M. tuberculosis* pre-incubated with anti-Ndk antibodies. Filter-associated radioactivity was quantified by liquid scintillation counter. Inset shows corresponding TLC.

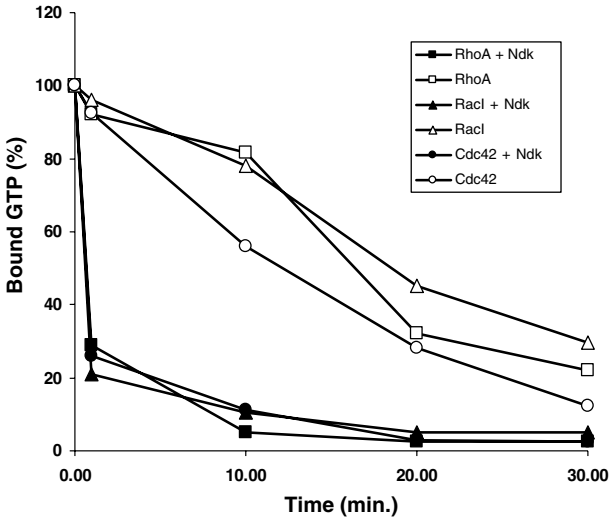

Fig. 3. Intrinsic and Ndk stimulated GTPase activity of RhoA, RacI and Cdc42. GSt-RhoA/RacI/Cdc42 proteins bound to glutathione–Sepharose beads were loaded with 10 μCi of [γ-^{32}P]GTP at 25 °C for 5 min and washed thrice. The [γ-^{32}P]GTP loaded Rho-GTPases were incubated with or without Ndk (1 μg) at 25 °C for different time points in a separate reaction mixture. At the end of incubation period, beads were washed twice, bound nucleotides were eluted, and radioactivity was measured with scintillation counter. Decrease in counts in the presence of Ndk is a measure of GAP activity of Ndk. Shown here is the remaining GTP at each time point.

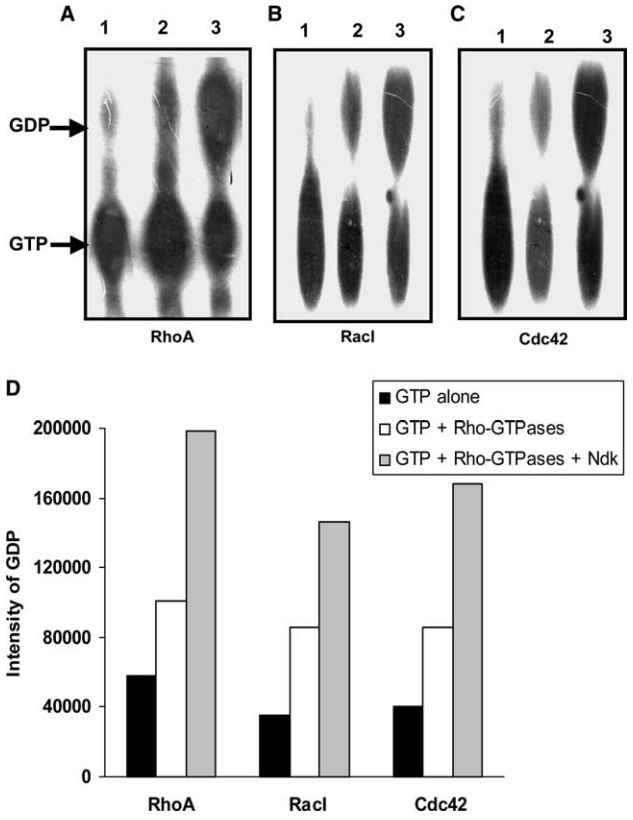

Fig. 4. GAP activity of Ndk with Rho-GTPases loaded with [α-^{32}P]GTP. GSH–Sepharose beads bound GST-Rho/Rac/Cdc42 proteins (1 μg each) were incubated with 3 μCi of [α-^{32}P]GTP at 25 °C for 5 min, beads were washed thrice and incubated with or without Ndk (0.5 μg) in separate reaction mixtures at 25 °C for 5 min. Beads were washed twice and bound nucleotides were eluted, resolved on PEI-TLC plates and visualized by an autoradiogram. (A–C) Lane 1, [α-^{32}P]GTP alone; Lane 2, [α-^{32}P]GTP plus Rho-GTPases (RhoA, RacI and Cdc42) and Lane 3, [α-^{32}P]GTP plus Rho-GTPases and Ndk. (D) Intensity of GDP formed was quantified by densitometer. Shown is the intensity of GDP formed when [α-^{32}P]GTP was loaded either with Rho-GTPases alone or in combination with Ndk.

It has been reported that Rho-GTPases control two distinct mechanisms of macrophages mediated phagocytosis. Type I mechanism utilizes immunoglobulin receptors and is governed by Cdc42 and Rac, whereas, type II phagocytosis is mediated by Rho and this process utilizes the complement receptors [4]. Rho-GAP proteins identified so far are known to differ in their specificity towards Rho family of GTPases. The substrate preference of Ndk for RhoA, RacI, and Cdc42 was analyzed in a time-dependent assay. It was observed that similar concentrations of Ndk stimulated the GTP-hydrolyzing activity for all GTPases (Fig. 3). These results indicated that Ndk increased the GTP-hydrolyzing activity of all three Rho proteins with almost similar efficiency. Similarly, ExoS of *P. aeruginosa* and YopE of *Y. pseudotuberculosis* have been shown to act as GAPs for Rho, Rac and Cdc42 [4,5]. Therefore, it can be speculated that GAP activity of Ndk could lead to modulation of the two major pathways of phagocytosis during *M. tuberculosis* infection. Similar speculation has also been made in the case of GAP activity of ExoS of *P. aeruginosa* [4].

Mechanism of Ndk mediated modulation of G-proteins activity is still not clearly understood. An earlier study suggests that Ndk does not interact directly with the G-proteins, rather Ndk influences the nucleotide state of several GTPases by acting on the nucleotide which dissociates from the GTPase during incubation [13]. In this study, two additional experiments were carried out to understand the mechanism of Ndk mediated conversion of Rho-bound GTP to Rho-GDP. In the first experiment, we observed that unlike with γ-phosphate-labeled Rho-GTPases, there was no loss of bound labeled phosphate when C. sup was incubated with α-phosphate labeled Rho-GTPases (Fig. 1B). In the second experiment, we examined the dissociation of bound nucleotides from Rho-GTPases loaded with [α-^{32}P]GTP in a time-dependent assay in the presence of purified Ndk. We observed that there was no dissociation of nucleotides that were bound to Rho-GTPases, as quantity of bound nucleotides present at the start and end of 30 min incubation, remained the same (Fig. 5). These results collectively suggest that Ndk of *M. tuberculosis* interacts directly with Rho-bound GTP and converts it to an inactive Rho-bound GDP form. Physiological significance of Ndk associated GAP activity is still not known. Several bacterial pathogens induce membrane ruffling and actin cytoskeleton rearrangement to promote their internalization by delivering GAP proteins into the host cell cytoplasm. In a recent study, it has been shown that internalization of *M. tuberculosis* in non-phagocytic cells induces membrane ruffling and cytoskeleton rearrangement [20]. This study tempted us to speculate that Ndk associated GAP activity might help in the internalization of *M. tuberculosis* in the host cells by inducing cytoskeleton reorganization as reported for other pathogens such as *S. typhimurium*, *Y. pseudotuberculosis* and *P. aeruginosa* [3–5].

3.4. Catalytic mutant of Ndk also has GAP activity

We have reported that Ndk of *M. tuberculosis* also has GTP binding and hydrolyzing activity and one of the mutants of

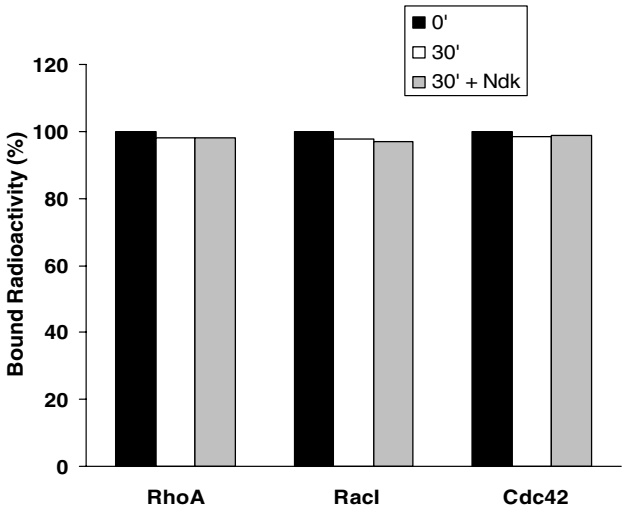

Fig. 5. GTP does not dissociate from the Rho-GTPases. GST-Rho/Rac/Cdc42 proteins were loaded with 10 μCi of [α-^{32}P]GTP at 25 °C for 30 min, spotted on nitrocellulose membrane and washed thrice. The [α-^{32}P]GTP loaded Rho-GTPases were incubated with or without purified Ndk at 25 °C for 30 min, membranes were washed, dried and quantified by liquid scintillation counter. Shown is the remaining bound α-^{32}P guanine nucleotide counts after 30 min in the presence and absence of Ndk.

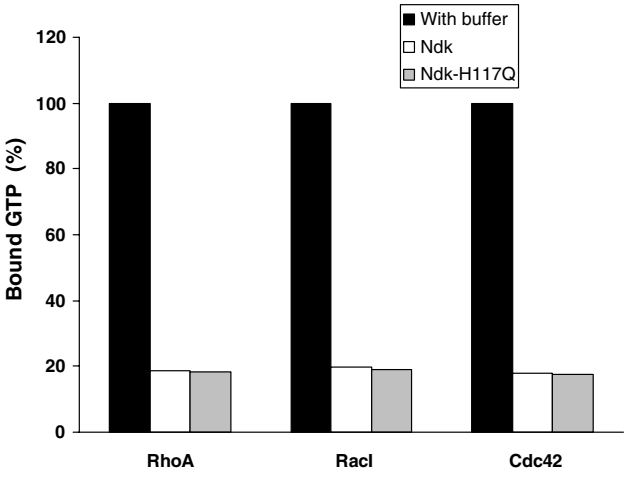

Fig. 6. Catalytic mutant of Ndk also has GAP activity. Effect of catalytic mutant of Ndk (H117Q) was also examined on the intrinsic GTPase activity of Rho-GTPase. Shown is the remaining bound GTP in the presence of Ndk and its mutant.

Ndk (H117Q) lacks both GTP binding and hydrolyzing activity [8]. In an earlier study, it has been shown that H118 mutant of Nm23H1 was deficient in GAP activity towards Rad family of GTPases [17]. Surprisingly, we observed that H117Q mutant of Ndk was also able to convert Rho-GTP into Rho-GDP (Fig. 6). This observation suggests that histidine at 117 position is not required for the structural stability or interaction with Rho-GTPases, as suggested earlier [17]. This observation also rules out the possibility that GTPase activity of Ndk may account for the observed GAP activity of Ndk for Rho-GTPases. These results also suggest that kinase and GAP activities of Ndk are not linked and appear to be mediated by different residues.

Therefore, in summary our results suggest that Ndk of *M. tuberculosis* acts as GAP for members of Rho-GTPases. Experiments are in progress to examine the in vivo role of Ndk in the Rho-GTPases mediated signaling pathways.

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