

Cytotoxic activity of nucleoside diphosphate kinase secreted from *Mycobacterium tuberculosis*

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Pathogenicity of *Mycobacterium tuberculosis* is closely related to its ability to survive and replicate in the hostile environment of macrophages. For some pathogenic bacteria, secretion of ATP-utilizing enzymes into the extracellular environment aids in pathogen survival via P2Z receptor-mediated, ATP-induced death of infected macrophages. A component of these enzymes is nucleoside diphosphate kinase (Ndk). The *ndk* gene was cloned from *M. tuberculosis* H₃₇Rv and expressed in *Escherichia coli*. Ndk was secreted into the culture medium by *M. tuberculosis*, as determined by enzymatic activity and Western blotting. Purified Ndk enhanced ATP-induced macrophage cell death, as assayed by the release of [¹⁴C]adenine. A catalytic mutant of Ndk failed to enhance ATP-induced macrophage cell death, and

periodate-oxidized ATP (oATP), an irreversible inhibitor of P2Z receptor, blocked ATP/Ndk-induced cell death. Purified Ndk was also found to be autophosphorylated with broad specificity for all nucleotides. Conversion of His117→Gln, which is part of the nucleotide-binding site, abolished autophosphorylation. Purified Ndk also showed GTPase activity. Collectively, these results indicate that secreted Ndk of *M. tuberculosis* acts as a cytotoxic factor for macrophages, which may help in dissemination of the bacilli and evasion of the immune system.

Keywords: cytotoxic; *Mycobacterium*; nucleoside diphosphate kinase; tuberculosis; GTPase.

Mycobacterium tuberculosis, the causative agent of tuberculosis, normally replicates in host macrophages. The pathogen has evolved several mechanisms to circumvent the hostile environment of macrophages. These include, (a) inhibition of phagosome–lysosome fusion [1], (b) inhibition of phagosomal acidification [2], (c) recruitment and retention of tryptophan/aspartate-containing coat protein on phagosomes to prevent their delivery to lysosomes [3], and (d) expression of members of the host-induced PE-PGRS family of proteins [4]. Another process that occurs with many bacterial pathogens concerns surface-associated P2Z receptors of macrophages. These receptors are involved in the killing of infected macrophages via external ATP that is effluxed from macrophages after activation by the invading pathogen [5]. A component of this system is the bacterial ATP-utilizing enzymes, that are secreted by bacterial pathogens such as, *Pseudomonas aeruginosa* [6,7], *Vibrio cholerae* [8], *Burkholderia cepacia* [9], and from *Trichinella spiralis*, an intracellular, parasitic nematode [10]. Culture

supernatant from *P. aeruginosa*, *V. cholerae*, and *B. cepacia*, harboring Ndk and other ATP-utilizing enzymes, is cytotoxic for macrophages and mast cells when ATP is present at millimolar concentrations [7–9]. Ndk is also secreted by the nonpathogenic bacterium *M. bovis* BCG [11], but addition of culture supernatant of *M. bovis* BCG prevents ATP-mediated cell death [11]. The culture supernatant of *M. bovis* BCG also contains an ATPase that can modulate ATP concentrations. As studies on Ndk have been performed using culture supernatant, the role of Ndk alone in the cytotoxicity process is not well understood.

In the present study, Ndk from *M. tuberculosis* was expressed in *E. coli* and purified. Antiserum elicited by the purified protein was used to show that Ndk is secreted from *M. tuberculosis*. Purified Ndk enhanced the cytotoxic effect of ATP on mouse macrophages. Further characterization of Ndk revealed the presence of GTPase and GTP-binding activities. Ndk, that probably functions as part of nucleotide metabolism, may contribute to pathogenicity by facilitating the destruction of host cells when secreted by *M. tuberculosis*.

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Abbreviations: ADC, albumin–dextrose complex; Ndk, nucleoside diphosphate kinase; Ni-NTA, nickel nitrilotriacetic acid; oATP, periodate-oxidized ATP; LPS, lipopolysaccharide. (Received 11 September 2002, revised 9 November 2002, accepted 27 November 2002)

Experimental procedures

Materials

Biochemicals, reagents and chromatography materials were purchased from Sigma Chemicals. Bacterial culture media and albumin–dextrose complex (ADC) were purchased from Difco Laboratories (BBL-Difco, Becton Dickinson,

New Delhi, India). Affinity resin (nickel nitrilotriacetic acid; Ni-NTA) was purchased from Qiagen. DNA modifying enzymes were obtained from New England Biolabs. Enhanced chemiluminescence (ECL) reagent and [¹⁴C]adenine (uniformly labeled) were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). [γ -³²P]ATP, [γ -³²P]GTP and [α -³²P]GTP were purchased from BRIT (Hyderabad, India).

Cell culture and preparation of culture supernatant

The J774A.1 macrophage cell line was maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 50 $\mu\text{g}\cdot\text{mL}^{-1}$ gentamycin sulfate (Life Technologies Gaithersburg, MD, USA).

M. tuberculosis H₃₇R_v (obtained from Dr J. S. Tyagi, AIIMS, N. Delhi, India) was grown in Middlebrook 7H9 medium supplemented with 10% ADC and 0.2% tween 80 at 37 °C with shaking at 220 r.p.m. for 3–4 weeks. The mid log-phase culture supernatant was filtered through a 0.22- μm filter and concentrated 50-fold using Centricon-10 concentrators (Millipore).

Plasmid construction and mutagenesis

M. tuberculosis genomic DNA was used as a template for PCR-based amplification of the Rv2445c gene, which encodes Ndk. The nucleotide sequence of two primers were: 5'-CTA GTG TTG GGA TCC GTG ACC GAA-3' carrying a *Bam*HI site at the 5' end (forward primer) and 5'-TCG GCG CAC AAG CTT CTA GGC GCC-3', that carried a *Hind*III site (reverse primer). The amplified product was digested with *Bam*HI and *Hind*III, and the resulting fragment was inserted into pQE-30 plasmid (Qiagen), which was previously digested with the same restriction enzymes. The recombinant plasmid was designated as pNdk.

Site-directed mutagenesis of His49, -53 and -117→Gln was performed by overlapping PCR. The oligonucleotides used included a forward primer 5'-CAC CAT CAC GGA TCC GTG ACC GAA-3', carrying *Bam*HI at its 5'-end and a reverse primer 5'-TCC GGA TGA GCA TTC ATC AGG-3'. The internal primers were 5'-GCC AGC CAG CAA TAC GCC GAA-3' and 5'-TTC GGC GTA TTG CTG GCT GGC-3' for mutation at position 49; 5'-TAC GCC GAA CAG GAA GGC AAA-3' and 5'-TTT GCC TTC CTG TTC GGC GTA-3' for mutation at position 53 internal primers were 5'-C AAC CTG GTG CAG GGG TCT G-3' and 5'-C AGA CCC CTG CAC CAG GTT G-3' for mutation at position 117 (underlined bases indicate His to Gln codon changes).

Purification of Ndk Protein

Ndk protein was purified as described previously [12]. In brief, *E. coli* SG13009 (pREP4) was transformed with recombinant plasmid pNdk. *E. coli* carrying recombinant plasmid was grown in Luria broth containing 100 μg of ampicillin and 25 μg of kanamycin per mL at 37 °C with shaking at 250 r.p.m. When D_{600} reached 0.6, isopropyl-1-thio- β -D-galactopyranoside was added to a final concentra-

tion of 1 mM. After 5 h of induction, the cells were harvested at 5000 *g*. For purification of protein, 1 L of culture pellet was resuspended in 20 mL of sonication buffer (50 mM NaP_i at pH 7.8 and 300 mM NaCl). Lysozyme (1 $\text{mg}\cdot\text{mL}^{-1}$) was added to the slurry followed by incubation on ice for 30 min. Phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM. Cells were sonicated at 4 °C (1 min burst, 1 min of cooling, 200–300 W) for five cycles. The resulting cell lysate was centrifuged at 15 000 *g* for 30 min. The supernatant fluid was mixed with 4 mL of Ni-NTA resin equilibrated previously with sonication buffer. The slurry was packed into a column and allowed to settle. The matrix was washed first with sonication buffer followed by wash buffer (50 mM NaP_i at pH 6.0, 500 mM NaCl and 10% glycerol). Protein was eluted with a linear gradient of 15 mL each of 0 and 500 mM imidazole chloride in elution buffer (50 mM NaP_i at pH 7.0, 100 mM NaCl and 10% glycerol). Fractions of 1 mL were collected and analyzed by 15% SDS/PAGE. The fractions containing purified Ndk were pooled.

Autophosphorylation assay

Autophosphorylation activity of the purified Ndk and mutant proteins were measured as described previously [13]. In brief, 1 μg of the purified Ndk or mutant proteins were incubated with 10 μCi of [γ -³²P]ATP or [γ -³²P]GTP (3000 Ci·mmol⁻¹) in a final reaction volume of 20 μL prepared with TMD buffer (50 mM Tris/HCl, 10 mM MgCl₂ and 1 mM of dithiothreitol, pH 7.4). The reaction was allowed to continue for 10 min and was terminated by the addition of 2 μL of 10% SDS. The samples were boiled for 10 min and separated by 15% SDS/PAGE. Analysis was by autoradiography.

Enzymatic activity of Ndk

Enzymatic activity of purified Ndk or its activity in culture supernatant of *M. tuberculosis* was assayed as described previously [14]. In brief, 1 μg of purified protein was incubated with 1 mM (final concentration) of each of NDP (where N is G, C or U) and 10 μCi of [γ -³²P]ATP (3000 $\mu\text{Ci}\cdot\text{mmol}^{-1}$) along with 0.1 mM ATP or with NDP (where N is A, C or U) and 10 μCi of [γ -³²P]GTP (3000 $\mu\text{Ci}\cdot\text{mmol}^{-1}$) along with 0.1 mM GTP, in a final volume of 20 μL of TMD buffer. The reaction was initiated by the addition of ATP or GTP and continued for 10 min at room temperature. Then, 2 μL of 10 \times SDS sample buffer was added. One μL of the reaction mixture was spotted onto a polyethyleneimine-thin layer chromatography (PEI-TLC) plate using 0.75 M KH₂PO₄ as the moving phase and visualized by autoradiography [14].

Production of polyclonal anti-Ndk Ig

Purified Ndk protein (50 μg) was solubilized in 500 μL of Freund's incomplete adjuvant and injected into Swiss albino mice. Subsequently, three injections of 25 μg each of Ndk in 250 μL of Freund's incomplete adjuvant were given after an interval of 14 days. Ten days after the final injection, animals were bled, and the titer of Ndk antiserum was determined by enzyme-linked immunosorbent assay (ELISA).

GTPase assay

Three methods were used to determine the GTPase activity associated with purified Ndk and in the culture supernatant of *M. tuberculosis*. In one [15], GTP hydrolysis was measured after purified Ndk (1 µg) was incubated with 1.0 µCi of [γ - 32 P]GTP in 20 µL of reaction volume in TMD buffer for different times at 25 °C. The reaction was terminated by addition of 2 µL of 4% SDS solution, and the reactants were resolved by polyethyleneimine thin layer chromatography (PEI-TLC) using 0.75 M KH_2PO_4 (pH 3.75). The decrease in the amount of [γ - 32 P]GTP was determined by the increase in the amount of the $^{32}\text{P}_i$. The same procedure was used for the ATPase assay.

In the second method [16], GTPase activity was determined after purified Ndk (1 µg) was mixed with 10 µCi of [γ - 32 P]GTP in 20 µL of buffer (20 mM Tris/HCl (pH 7.6), 5 mM EDTA, 1 mM dithiothreitol) and 3 µL of mix was diluted 10 times using dilution buffer (20 mM Tris, pH 7.6, 0.1 mM dithiothreitol, 1 mM GTP and BSA 1 mg·mL $^{-1}$). Diluted mix (5 µL) was removed (0 min) and further incubated for different times at room temperature. Then, 5 µL of samples were removed and spotted on nitrocellulose filters (Millipore), washed extensively with cold assay buffer and air-dried. Filter-associated radioactivity was determined by liquid scintillation counter.

In the third method [17], GTPase activity was measured after purified Ndk (1 µg) was incubated with 3 µCi of [α - 32 P]GTP in a buffer consisting of 50 mM Tris/HCl (pH 7.4), 1 mM MgCl_2 , 1 mM dithiothreitol and 1 mg·mL $^{-1}$ bovine serum albumin at 25 °C for 10 min. The reaction was stopped by addition of 4 µL of 4× SDS sample buffer. Reaction mixture (1 µL) was loaded onto the PEI-TLC plate to resolve GTP and GDP. Analysis was by autoradiography.

GTP binding assay

GTP binding assay was measured by the nitrocellulose filter binding method as described previously [15]. Binding was carried out in TMD buffer. One microgram of the purified protein was spotted on the nitrocellulose filter paper (2 × 2 cm), air dried for 10 min and placed in a Petriplate with 10 mL of TMD buffer containing 1 µCi of [γ - 32 P]GTP (3000 Ci·mmol $^{-1}$). The binding reaction was carried out for various times at 25 °C. After completion of the binding reaction, each filter was washed several times with an excess of TMD buffer, air dried and autoradiographed.

Cytotoxicity assay

Cytotoxic activity of purified Ndk and concentrated culture supernatant of *M. tuberculosis* were measured as described earlier [8,18]. Macrophages (J774A.1) were cultured in a 12-well tissue culture plate in 1 mL of DMEM media supplemented with 10% fetal bovine serum and incubated overnight at 37 °C in a CO $_2$ incubator (5% CO $_2$). Cells were labeled with [14 C]adenine by adding media containing 1 µCi·mL $^{-1}$ for 6 h. The labeled cells were washed three times with the same medium to remove unincorporated [14 C]adenine. Cells were incubated with medium containing 50 ng of lipopolysaccharide (LPS) per mL for 12 h. LPS-

primed cells were washed three times and incubated with 3 mM of ATP with or without purified Ndk or *M. tuberculosis* culture supernatant for different times. At the end of each incubation, 150 µL of supernatant was aspirated from each well and radioactivity was determined by liquid scintillation counting. In experiments with P2Z receptor antagonist, macrophages were preincubated with 1 mM of periodate oxidized ATP (oATP) for 2 h prior to addition of ATP.

Results

Expression and purification of Ndk

M. tuberculosis gene Rv2445c (Ndk) was amplified by PCR from genomic DNA of *M. tuberculosis* H $_37$ Rv and cloned into the pQE30 expression plasmid. The resulting plasmid, designated as pNdk, was transferred to *E. coli* SG13009 (pREP4) by bacterial transformation, and the Ndk protein was purified using Ni-NTA affinity matrix chromatography. The protein migrated with an apparent molecular mass of 14.4 kDa during 15% SDS/PAGE (Fig. 1). This result was consistent with the calculated molecular mass of Ndk.

Ndk is defined by its ability to catalyze the transfer of terminal phosphate from any NTP to any NDP. Enzyme activity was assayed by incubating purified protein with [γ - 32 P]ATP and 1 mM of unlabelled G-, U- or CDP or [γ - 32 P]GTP and 1 mM of A-, C- or UDP. After 10 min incubation at room temperature, the mixture was separated by PEI-TLC. As shown in Fig. 2A and 2B, Ndk transferred

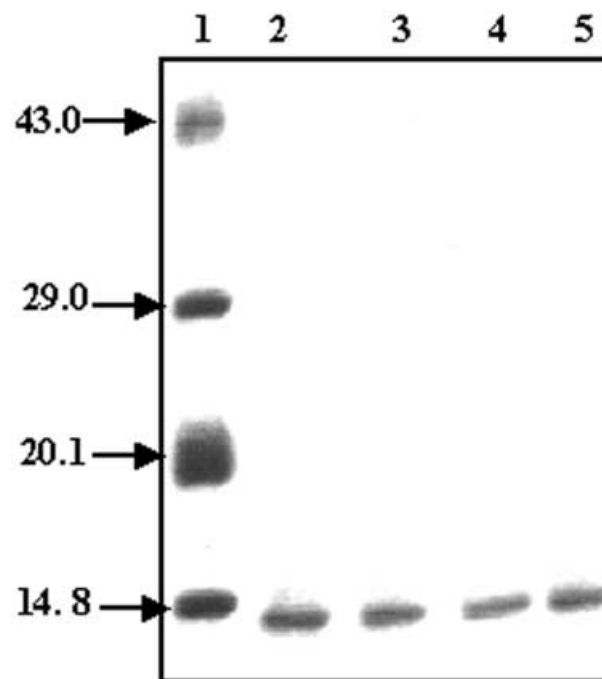


Fig. 1. Electrophoretic analysis of recombinant pNdk and mutants. Affinity purified Ndk and mutant proteins (2 µg) were separated by 15% SDS/PAGE and stained with coomassie blue. Lane: 1, molecular mass marker; lane 2, Ndk; lane 3, Ndk H49Q; lane 4, Ndk H53Q and lane 5, Ndk H117Q.

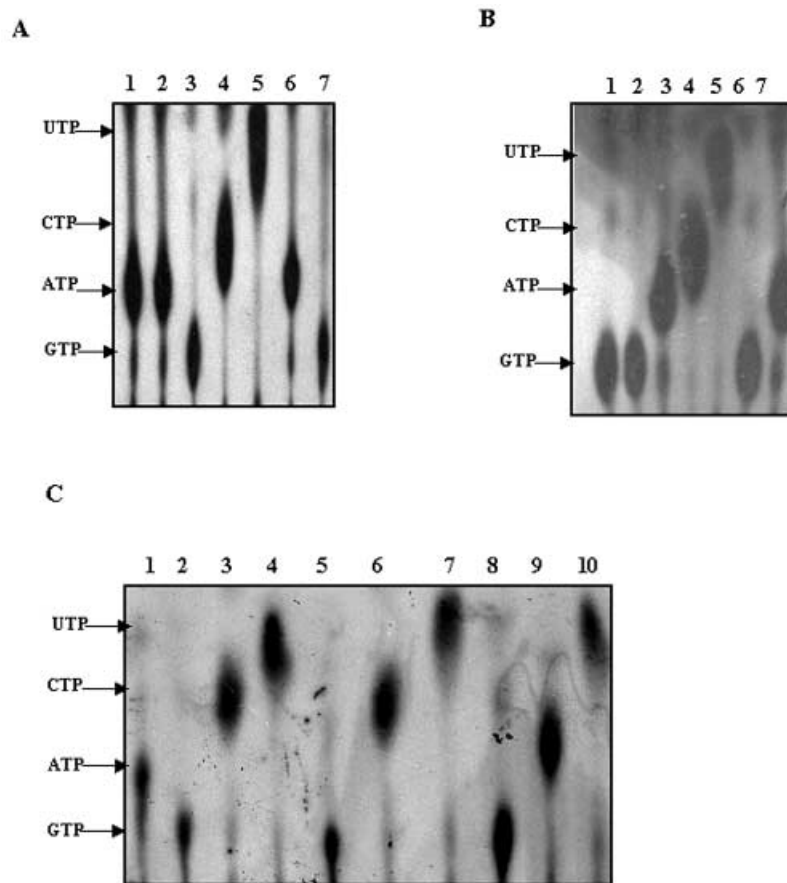


Fig. 2. Nucleoside diphosphate kinase activity of Ndk. Purified Ndk and mutant proteins (1 μg) were incubated with 10 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and 1 mM NDP (G-, C- or UDP) or $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ and 1 mM NDP (A-, C- or UDP) for 10 min at room temperature. Reaction was stopped by the addition of 2 μL of 10 \times SDS/PAGE buffer and resolved by PEI-TLC. (A) Experiment with Ndk and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$: (Lane 1, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ control; lane 2, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ plus GDP; lane 3, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ plus GDP and Ndk; lane 4, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ plus CDP and Ndk; lane 5, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ plus UDP and Ndk; lane 6, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ plus UDP and heat inactivated Ndk; lane 7, $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ as a control). (B) Experiment with Ndk and $[\gamma\text{-}^{32}\text{P}]\text{GTP}$: (Lane 1, $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ control; lane 2, $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ plus ADP; lane 3, $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ plus ADP and Ndk; lane 4 $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ plus CDP and Ndk; lane 5, $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ plus UDP and Ndk; lane 6, $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ plus UDP and heat inactivated Ndk; lane 7, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as a control). (C) Experiment with His mutants (pNdk H49Q, H53Q and H117Q) of Ndk with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$: (Lane 1, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ control; lane 2, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ plus GDP and H49Q; lane 3, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ plus CDP and H49Q; lane 4, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ plus UDP and H49Q; lane 5, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ plus GDP and H53Q; lane 6, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ plus CDP and H53Q; lane 7, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ plus UDP and H53Q; lane 8, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ plus GDP and H117Q; lane 9, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ plus CDP and H117Q; lane 10, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ plus UDP and H117Q).

a terminal phosphate from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ or $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ to all NDP, converting them to the corresponding triphosphates. Heat inactivated (100 $^{\circ}\text{C}$ for 10 min) purified Ndk failed to show phosphotransferase activity (Fig. 2A and 2B).

In *M. tuberculosis*, Ndk contains His at amino acid positions 49, 53 and 117. Each His was replaced individually with Gln by overlapping PCR. The resulting mutant plasmids were designated as pNdk H49Q, pNdk H53Q and pNdk H117Q. Mutant proteins were purified by Ni-NTA affinity matrix chromatography and assayed for enzymatic activity. All the mutants showed similar phosphotransferase activity as that of native Ndk (Fig. 2C).

ATPase activity of purified Ndk

Purified Ndk, and mutant proteins (H49Q, H53Q and H117Q) were also analyzed for their ability to bind and

hydrolyze ATP. Purified Ndk showed ATPase activity as evidenced by the decrease in amount of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and the simultaneous increase in $^{32}\text{P}_i$ (Fig. 3). The activities of two mutants (H49Q and H53Q) were similar to those of wild-type Ndk. However, mutation at position 117 (H117Q) resulted in loss of both ATP binding and hydrolysis activity (Fig. 3). Thus, H117 is crucial for ATPase activity.

Secretion of nucleoside diphosphate kinase by *M. tuberculosis*

M. tuberculosis H₃₇Rv culture supernatant exhibited Ndk activity when assayed by transfer of terminal $\gamma\text{-}^{32}\text{P}$ from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ or $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ to any of the four NDP (Fig. 4A, 4B).

To confirm that Ndk was secreted from *M. tuberculosis* H₃₇Rv, proteins of concentrated, mid log-phase culture

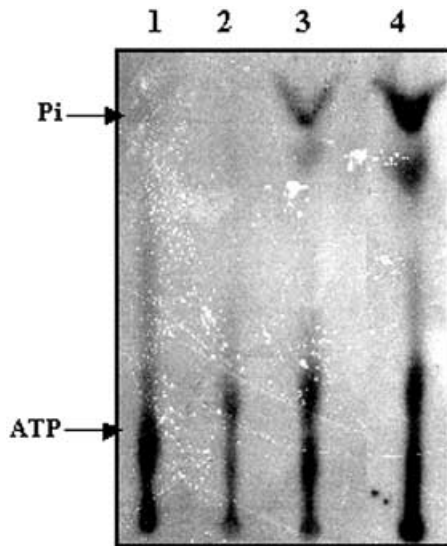


Fig. 3. ATPase activity in Ndk of *M. tuberculosis*. Purified Ndk and mutant H117Q were incubated with 10 μ Ci of [γ - 32 P]ATP at 25 $^{\circ}$ C for various time periods and release of 32 P_i was monitored as an indicator of ATPase activity. Lane 1, [γ - 32 P]ATP; lane 2, [γ - 32 P]ATP plus H117Q at 30 min; lane 3, [γ - 32 P]ATP plus Ndk at 15 min; lane 4, [γ - 32 P]ATP plus Ndk at 30 min.

supernatant were separated by SDS/PAGE, transferred to nitrocellulose, and probed with immune serum prepared from mice injected with purified, recombinant Ndk. The presence of Ndk was observed in the culture supernatant (Fig. 5A). In contrast, adenylate kinase (a cytoplasmic protein) was not detected by Western blot using polyclonal antibody against purified adenylate kinase (Fig. 5B).

Autophosphorylation activity

The autophosphorylating activity of Ndk was determined by incubating purified protein with [γ - 32 P]ATP at room temperature for 5 min. Proteins were separated by 15% SDS/PAGE and analyzed by autoradiography. A sharp band at 14.4 kDa was observed, indicating that Ndk is an autophosphorylating enzyme (Fig. 6A). Both the H49Q and H53Q mutant proteins were autophosphorylated, while the H117Q Ndk protein was not (Fig. 6A). These data indicate that in Ndk of *M. tuberculosis* H117 is required for autophosphorylation. The presence of native and mutant Ndk protein in each reaction was shown by Western blot using anti-Ndk antibodies (Fig. 6B).

GTPase activity

We next examined the ability of Ndk to bind and hydrolyze GTP by three methods. In the first, Ndk was incubated with [γ - 32 P]GTP for various times at 25 $^{\circ}$ C. A time-dependent increase in 32 P_i formation and decrease in [γ - 32 P]GTP was observed that was proportional to Ndk concentration (Fig. 7A). Second, Ndk-associated GTPase activity was demonstrated in a filter-binding assay by incubating Ndk with [γ - 32 P]GTP which resulted in hydrolysis of 60% bound GTP in 30 min (Fig. 7B). Third, GTPase activity was

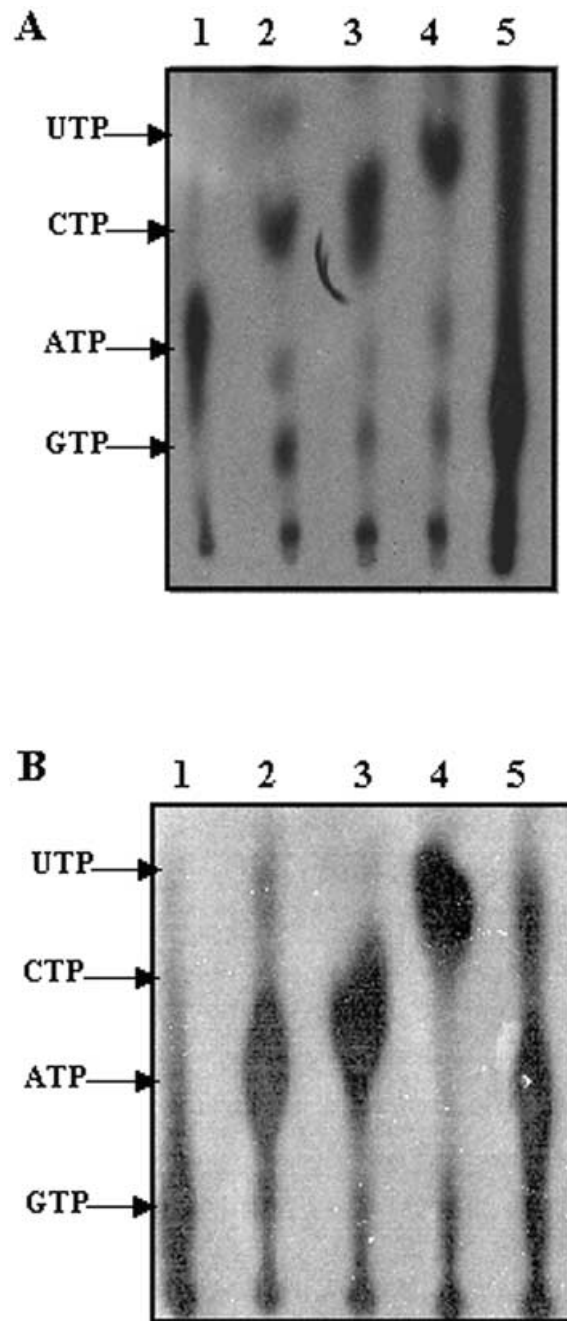


Fig. 4. Ndk activity in the supernatant of *M. tuberculosis* culture. *M. tuberculosis* was grown in 7H9 media and mid log-phased cells were harvested. Culture supernatant was filtered through 0.22 μ m filter and concentrated 50-fold by Centricon and filtrate was used for the enzyme assay as described in the experimental procedure. Culture supernatant (10 μ L) was incubated with 10 μ Ci of [γ - 32 P]ATP and 1 mM NDP (G, C or UDP) or [γ - 32 P]GTP and 1 mM NDP (A, C or UDP) for 10 min at room temperature. Reaction was stopped by the addition of 2 μ L of 10 \times SDS/PAGE buffer and resolved by PEI-TLC. (A) Experiment with [γ - 32 P]ATP: (Lane 1, [γ - 32 P]ATP control; lane 2, [γ - 32 P]ATP plus GDP; lane 3, [γ - 32 P]ATP plus CDP; lane 4, [γ - 32 P]ATP plus UDP; and lane 5, [γ - 32 P]GTP as a control). (B) Experiment with [γ - 32 P]GTP: (Lane 1, [γ - 32 P]GTP control; lane 2, [γ - 32 P]GTP plus ADP; lane 3, [γ - 32 P]GTP plus CDP; lane 4 [γ - 32 P]GTP plus UDP; and lane 5, [γ - 32 P]ATP as a control).

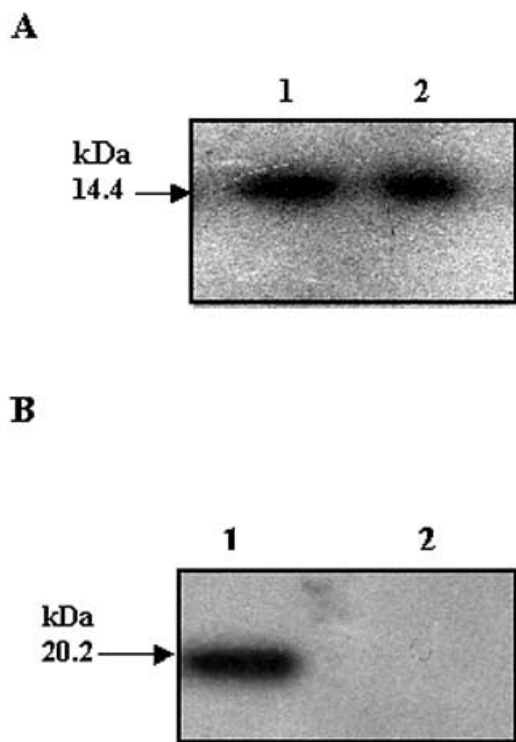


Fig. 5. Western blot analysis of culture supernatant of *M. tuberculosis*. Concentrated culture supernatant, purified Ndk and adenylate kinase were separated on 15% SDS/PAGE, proteins were transferred to a nitrocellulose membrane incubated with anti-Ndk (A) or anti-adenylate kinase antibodies (B) and developed with ECL reagent. Lane 1, purified Ndk or adenylate kinase and lane 2, culture supernatant.

measured by incubating purified Ndk with [α - 32 P]GTP for 10 min followed by separation of the products by PEI-TLC to observe the formation of [α - 32 P]GDP (Fig. 7C). Ndk was bound to [γ - 32 P]GTP in a time-dependent fashion, suggesting that binding of GTP to Ndk is important for its GTPase activity (data not shown).

The H49Q, H53Q and H117Q mutant Ndk proteins were also analyzed for their ability to bind and hydrolyze GTP. The activities of two mutants (H49Q and H53Q) were similar to those of wild-type Ndk. However, mutation at position 117 (H117Q) resulted in loss of both GTP binding and GTP hydrolysis activity (Fig. 7C). Thus, H117 is crucial for both activities.

Enhancement of cytotoxic action by Ndk

Macrophages expel ATP upon activation by either bacterial LPS or intact bacteria [5]. The ATP then activates P2Z receptors on the surface of macrophages, which in turn trigger macrophage cell death by formation of large, nonselective membrane pores that are permeable to molecules up to a mass of 900 Da [19]. In the present study, ATP alone was cytotoxic to macrophages and resulted in the leakage of [14 C]adenine up to 29% in 8 h. Ndk, in combination with ATP, increased cytotoxicity in a time-dependent manner (Fig. 8A). Addition of purified Ndk to the macrophage cells, in combination with 3 mM ATP, resulted in 79% leakage of [14 C]adenine in 8 h. Ndk alone

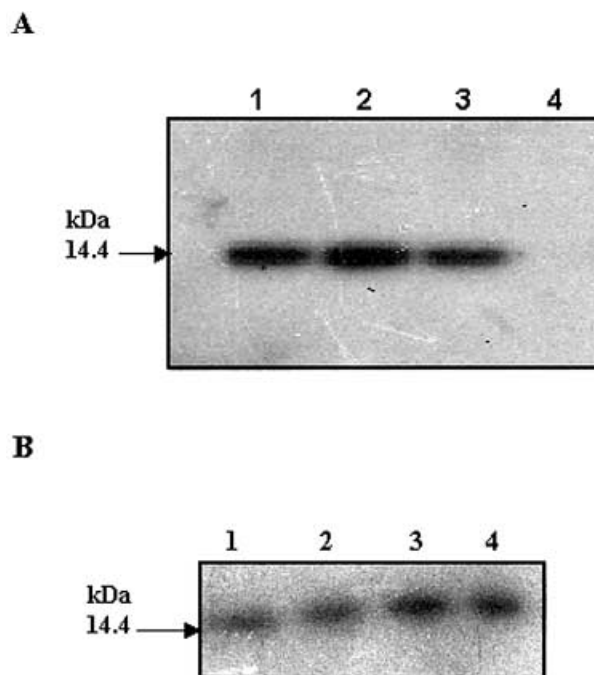


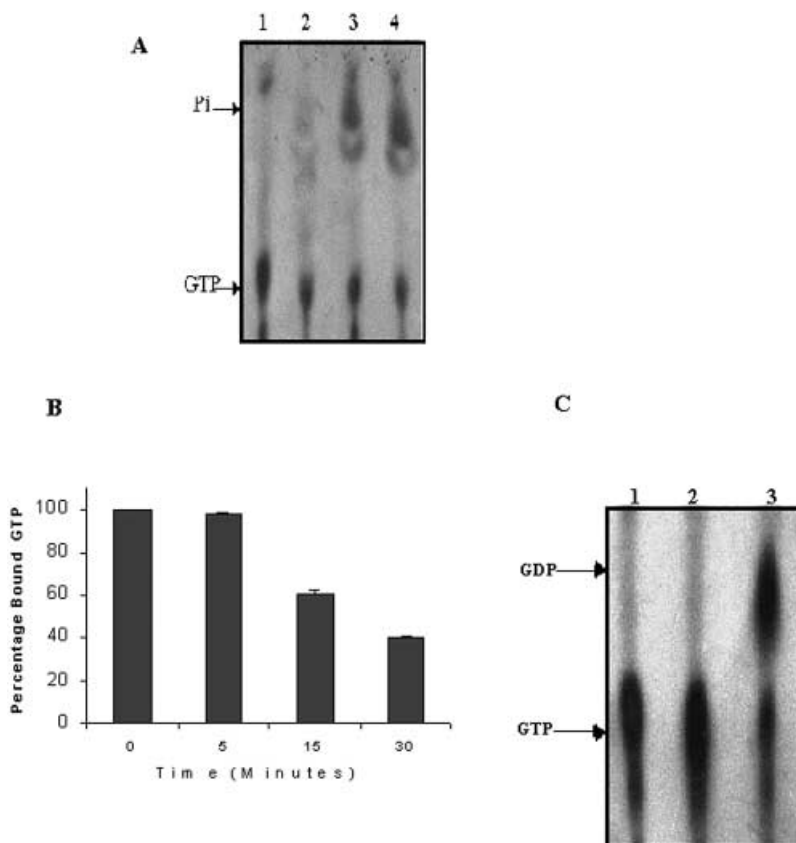
Fig. 6. Autophosphorylation of recombinant Ndk and mutant proteins. (A) Ndk and mutant proteins (1 μ g) were incubated in the presence of 10 μ Ci of [γ - 32 P]ATP in 20 μ L of reaction volume. The reaction was stopped by the addition of 2 μ L of 10% SDS/PAGE loading buffer. Fractions were resolved by 15% SDS/PAGE and autoradiographed. Lane 1, Ndk; lane 2, Ndk H49Q; lane 3, Ndk H53Q; lane 4, Ndk H117Q. (B) Detection of Ndk and three His mutants of Ndk by anti-Ndk antibody. Ndk and mutant proteins (1 μ g) were separated on 15% SDS/PAGE, proteins were transferred to nitrocellulose membrane. Probed with anti-Ndk antibody raised in mice and developed using ECL reagent. Lane 1, Ndk; lane 2, H49Q; lane 3, H53Q and lane 4, H117Q.

had no significant effect on release of [14 C]adenine. Mutant H117Q Ndk failed to stimulate ATP-dependent cytotoxicity (Fig. 8A). This result was expected, as the mutant also lacked ATP binding and ATP hydrolysis activity.

To further investigate the role of Ndk in ATP-mediated cytotoxicity, culture supernatant of *M. tuberculosis* H37Rv was examined for ATP-dependent cytotoxicity. Culture supernatant, in combination with 3 mM ATP, resulted in 48% leakage of [14 C]adenine in 5 h. Addition of anti-Ndk polyclonal antibody to the culture supernatant halted the ATP-mediated leakage of adenine (Fig. 8B). Cytotoxicity of purified Ndk was also measured in the presence of a mixture of 3 mM ADP and 1 mM each of G-, C- and UTP. It was observed that Ndk was cytotoxic to the macrophages in the presence of the mixture, while alone the mixture was not toxic (Data not shown).

As a test for involvement of surface P2Z receptors, we examined the effect of oATP, a well-known P2Z receptor antagonist [20]. When macrophages were pretreated with 1 mM oATP prior to the addition of ATP and Ndk, oATP prevented the ATP- and Ndk-induced leakage of [14 C]adenine (Fig. 8A). Thus, the cytotoxicity associated with purified Ndk appears to be mediated by the macrophage cell surface P2Z receptors.

Fig. 7. GTPase activity of purified Ndk. (A) [γ - 32 P]GTP hydrolysis. Purified Ndk (1 μ g) was incubated with 10 μ Ci of [γ - 32 P]GTP at 25 °C for various time periods (0–30 min), and release of 32 P_i was noted as an indicator of GTPase activity. Lane 1, [γ - 32 P]GTP alone; lane 2, [γ - 32 P]GTP plus Ndk at 5 min; lane 3, [γ - 32 P]GTP plus Ndk at 15 min; lane 4, [γ - 32 P]GTP plus Ndk at 30 min. (B) Filter binding assay. purified protein (1 μ g) was incubated with 10 μ Ci of [γ - 32 P]GTP for various time intervals (0–30 min). GTPase activity was analyzed by filter binding assay as described in the experimental procedure. Shown is the remaining GTP at each time points as percent of bound [γ - 32 P]GTP before incubation at 37 °C. (C) Hydrolysis of [α - 32 P]GTP. Purified Ndk (1 μ g) was incubated with 3 μ Ci of [α - 32 P]GTP, for 10 min and mixture was resolved by PEI-TLC and autoradiographed. Lane 1, [α - 32 P]GTP; lane 2, [α - 32 P]GTP incubated with H117Q, lane 3 [α - 32 P]GTP plus Ndk.



Discussion

The results presented above indicate that Ndk is secreted by *M. tuberculosis* as a cytotoxic factor that facilitates ATP-dependent P2Z receptor-mediated macrophage death. The Ndk gene was cloned and expressed in *E. coli*, and Ndk was purified as a His-tagged protein. Antibody was raised against purified Ndk in mice and used to study secretion of Ndk from *M. tuberculosis*. Western blot analysis of concentrated supernatant of *M. tuberculosis* suggested that Ndk is secreted in the culture media. In order to determine whether the detection of Ndk in the culture supernatant of *M. tuberculosis* H37Rv is caused by the secretion rather than by the autolysis of the cells, culture supernatant was also analysed for the presence of a cytoplasmic protein, adenylate kinase. Western blot analysis showed that adenylate kinase of *M. tuberculosis* was absent from the culture supernatant suggesting that the presence of Ndk in culture supernatant is due to secretion and not autolysis (Fig. 5B). Secretion of Ndk, a crucial enzyme of metabolism seems unusual, but its secretion has been reported from several organisms such as *P. aeruginosa*, *V. cholerae*, *B. cepacia*, *T. spiralis*, *M. bovis* and *M. smegmatis* [6–10,14]. Purified Ndk stimulated ATP-induced cytotoxicity in cultured murine macrophage cells (Fig. 8A). Thus, secreted Ndk from *M. tuberculosis*, like culture supernatant of *V. cholerae* and *B. cepacia* that harbors Ndk and other ATP-utilizing enzymes, acts as a cytotoxic virulence factor [8,9].

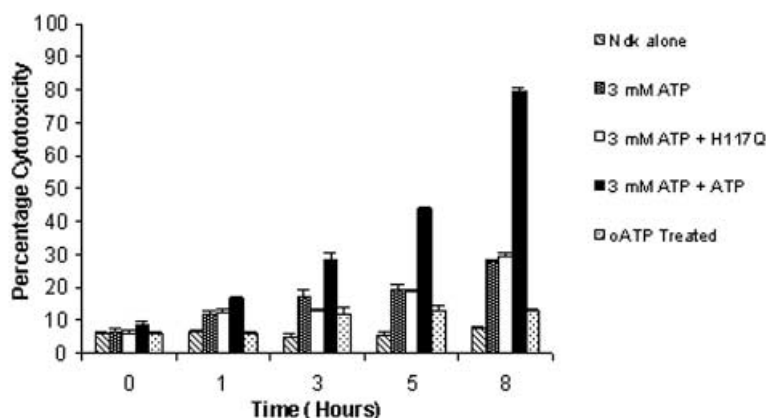
Ndk was also cytotoxic to macrophages in the presence of a mixture of ADP, G-, C- and UTP, while alone this mixture was less cytotoxic (data not shown). This observation

suggests that ADP was converted to ATP by Ndk through the transfer of a terminal phosphate from a pool of other triphosphates (C-, G- and UTP) present in the medium. It has been observed that different ionic forms of ATP and adenine nucleotides differ in their agonist activities towards P2Z receptor activation [19,21]. The enhancement in ATP-mediated cytotoxicity of Ndk as compared to ATP alone might be due to Ndk-mediated conversion of ATP into various adenine nucleotides that may act as better agonists than ATP itself. Such speculations have also been made in the cases of *P. aeruginosa*, *V. cholerae* and *B. cepacia* [7–9].

Pretreatment of macrophages with an antagonist of the P2Z receptor, oATP, protected the cells from Ndk-mediated cytotoxicity, suggesting that Ndk of *M. tuberculosis* acts via the P2Z receptors. The mechanism of Ndk-mediated cytotoxicity is ATP-mediated, as mutant H117Q, which is deficient in ATP binding and hydrolysis activities failed to stimulate ATP-mediated cytotoxicity (Fig. 8A).

Culture supernatant of *M. tuberculosis* was found to be cytotoxic to macrophages in the presence of 3 mM ATP. Addition of anti-Ndk polyclonal antibody resulted in a time-dependent decrease in ATP-mediated cytotoxicity of culture supernatant of *M. tuberculosis* H37Rv (Fig. 8B), suggesting that this cytotoxicity was induced by Ndk present in the culture supernatant. Several other intracellular pathogens, such as *Salmonella typhimurium*, *Legionella pneumophila* and *Listeria monocytogenes*, induce apoptosis in immune cells [22–24]. It has been suggested that the induction of programmed cell death before macrophages can synthesize pro-inflammatory cytokines may play an important role in bacterial evasion of the host immune system [22]. The ability

A



B

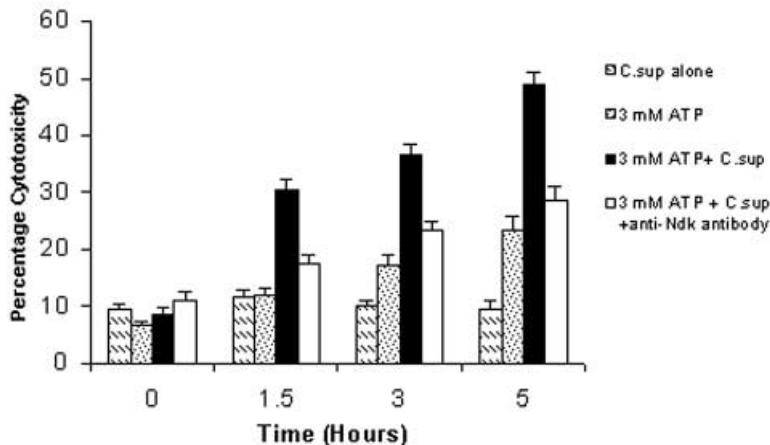


Fig. 8. ATP-induced macrophage cytotoxicity from purified Ndk and culture supernatant of *M. tuberculosis*. J774A.1 cells were labeled with [14 C]adenine ($1 \mu\text{Ci}\cdot\text{mL}^{-1}$) for 6 h and stimulated with LPS ($50 \text{ ng}\cdot\text{mL}^{-1}$) for 12 h. For the experiment with oATP, cells were pretreated with 1 mM oATP for 2 h before the cytotoxicity assay was carried out. Release of [14 C]adenine into media was counted using liquid scintillation counter. Each value is the average \pm SEM and representative of four experiments with duplicate wells for each treatment. (A) Experiment with purified Ndk ($25 \mu\text{g}\cdot\text{mL}^{-1}$) in presence or absence of exogenous ATP (3 mM). (B) Experiment with concentrated culture supernatant of *M. tuberculosis* in presence or absence of exogenous ATP (3 mM).

of *M. tuberculosis* to promote apoptosis may also be important for dissemination of infection. A knockout mutant of Ndk in *M. tuberculosis* would give important insight into the *in vivo* role of Ndk. Experiments are in progress to construct an *ndk* knockout mutant of *M. tuberculosis*.

The role of *M. tuberculosis* Ndk is to produce nucleoside triphosphates (NTP) as precursors for RNA, DNA and polysaccharide synthesis. Ndk catalyzes the reversible transfer of the 5'-terminal P_i from NTP to NDP [25]. The central importance of such a function is consistent with the failure of attempts to isolate knockout mutants of *ndk* in *Mycobacterium xanthus* [26]. However, in a few organisms, such as *E. coli* and *P. aeruginosa*, Ndk activity is complemented by adenylate kinase and pyruvate kinase [6,27]. Ndk also plays a vital role in the physiology of the eukaryotes. For example, in *Drosophila*, a null mutation in *ndk* causes abnormalities in larval development that lead to tissue necrosis and death at the prepupal stage [28]. Thus, Ndk might have multiple functions. In humans, reduction of *ndk* transcript level is associated with lowered metastatic potential in tumor cells [29]. In the present study it was observed that purified Ndk from *M. tuberculosis* was able to transfer terminal P_i both from [γ - 32 P]ATP and [γ - 32 P]GTP to all

nucleoside diphosphates and to convert them to their corresponding triphosphates (Fig. 2A and B). Ndk from *M. tuberculosis* is thermostable upto 75°C and becomes inactivated completely at 82°C [30]. In this study, heat inactivated Ndk (100°C , 10 min) was also checked for enzymatic activity and found to lack phosphotransferase activity (Fig. 2A and B).

All three His mutants of Ndk (pNdk-H49Q, H53Q and H117Q) showed similar phosphotransferase activity (Fig. 2C). The presence of phosphotransferase activity in mutant pNdk-H117Q was surprising, as this mutant lost both ATP-binding and hydrolysis activity (Fig. 3). Similar activity has been reported for the His mutant of Ndk from *Dictyostelium discoideum*. It has been shown that nucleophilic His can be rescued by other exogenous small nucleophiles including water [31,32].

Ndk is autophosphorylated, and His117 is the only His residue that is conserved in all known Ndk characterized to date [33]. In *Mycobacterium xanthus* it has been reported that replacement of His117 with Gln in Ndk abolishes the autophosphorylation and nucleotide binding activity [33]. Ndk of *M. tuberculosis* has three His residues at positions 49, 53 and 117 that were replaced individually with Gln.

Replacement of H117Q but not H49Q or H53Q resulted in the loss of both autophosphorylation and nucleotide binding activity (Figs 3, 6 and 7). Thus only His117 is critical for autophosphorylation and nucleotide binding.

In this report, we show that Ndk has intrinsic GTPase and GTP binding activity (Fig. 7A–C). *M. tuberculosis* Ndk lacks the GXXGK and DXG motifs that are characteristic features of GTP binding proteins [34,35]. The sequence, NKKD, which is known to be involved in guanine base recognition [36] is also absent from *M. tuberculosis* Ndk.

In summary, our results suggest that Ndk secreted by *M. tuberculosis* is a cytotoxic factor that induces ATP-dependent P2Z receptor-mediated macrophage death. In addition, we showed that Ndk has GTPase activity. The ability of *M. tuberculosis* to promote apoptosis may be important for the initiation of infection, bacterial survival, and escape of the host immune response.

Acknowledgements

We thank Prof. S. K. Brahmachari for making this work possible. P. C. and A. S. were supported by University Grant Commission (UGC), N. Delhi. We are also thankful to L. S Meena, P. K. Gupta, H. Chandra, H. Khanna Parampal, R. Gaur for valuable discussions and Vineet and Neeraj for helping with bioinformatics work. Financial support for the project was provided by NMITLI, Council of Scientific and Industrial Research (CSIR).

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