Secretory nucleoside diphosphate kinases from both intra- and extracellular pathogenic bacteria are functionally indistinguishable

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Nucleoside diphosphate kinase (NDK), responsible for the maintenance of NTP pools, is an ATP-utilizing enzyme secreted by different pathogens. We found that NDK from Salmonella enterica serovar Typhimurium (S. Typhimurium) is also secretory in nature. Secretory NDK is known to play a crucial role in the survival of pathogenic microbes within host cells through their interaction with extracellular ATP. To elucidate this aspect, we assessed the contribution of secretory products containing NDK from intracellular (Mycobacterium tuberculosis and S. Typhimurium) and extracellular (Vibrio cholerae) pathogens to the process of ATP-induced J774 mouse macrophage cell lysis by monitoring lactate dehydrogenase (LDH) release in the culture medium. Compared with an untreated control, our results demonstrate that S. Typhimurium secretory products caused a greater than twofold decrease in LDH release from J774 macrophage cells treated with ATP. Furthermore, the secretory products from an ndk-deleted strain of S. Typhimurium did not display such behaviour. Contrary to this observation, the secretory products containing NDK of V. cholerae were found to be cytotoxic to J774 cells. At the amino acid level, the sequences of both the NDKs (S. Typhimurium and V. cholerae) exhibited 65% identity, and their biochemical characteristics (autophosphorylation and phosphotransfer activities) were indistinguishable. However, to our surprise, the secretory product of an ndk-deleted strain of S. Typhimurium, when complemented with V. cholerae ndk, was able to prevent ATP-induced cytolysis. Taken together, our results unambiguously imply that the intrinsic properties of secretory NDKs are identical in intra- and extracellular pathogens, irrespective of their mode of manifestation.

INTRODUCTION

Host cells infected by pathogens usually accumulate extracellular ATP (eATP) at the site of inflammation as a part of their defence mechanism (Dubyak & el-Moatassim, 1993; Ferrari et al., 1997). The eATP, depending on the duration of exposure to cells, binds to purinergic receptors, particularly P2X7 (Chiozzi et al., 1996; Di Virgilio, 1995), and causes cytolysis via necrotic or apoptotic pathways (Blanchard et al., 1995; Lammas et al., 1997; Molloy et al., 1994; Zaborina et al., 1999a; Zambon et al., 1994). In intracellular pathogens, such as Mycobacterium tuberculosis, Porphyromonas gingivalis and Trichinella spiralis, secretory ATP-utilizing enzymes have been shown to use eATP as their principal substrate (Chopra et al., 2003; Gounaris et al., 2001; Yilmaz et al., 2008; Zaborina et al., 1999a) and thus prevent host cell death. On the other hand, extracellular pathogens such as Pseudomonas aeruginosa and Vibrio cholerae have developed strategies to avoid the phagocytic activity of macrophages by releasing enzymes which increase levels of eATP and as a result trigger apoptosis of professional phagocytes (Punj et al., 2000; Zaborina et al., 2000). Thus, it is apparent that the physiological requirements of intracellular and extracellular pathogens are distinct. This has led to the hypothesis that intra- and extracellular pathogens develop strategies in opposite directions (either avoiding or promoting the phagocytic activity of macrophages and/or other professional phagocytes) by releasing secretory ATP-utilizing enzymes which either increase or decrease the level of eATP.

Available literature indicates that secretory enzymes of different pathogens participating in ATP utilization include nucleoside diphosphate kinase (NDK), adenylate kinase, 5’ nucleotidase, ecto-ATPase and ecto-nucleoside triphosphate

Abbreviations: Bz-ATP, benzyl-benzyl ATP; eATP, extracellular ATP; GST, glutathione S-transferase; LDH, lactate dehydrogenase; mNDK, M. tuberculosis NDK; NDK, nucleoside diphosphate kinase; O-ATP, oxidized ATP; PEL, polyethyleneimine; sNDK, S. Typhimurium NDK; vNDK, V. cholerae NDK.

Three supplementary figures and a supplementary table are available with the online version of this paper.
diphosphohydrolase (Berrêdo-Pinho et al., 2001; Pinheiro et al., 2006). Among these, NDK is usually a cytoplasmic enzyme, present in virtually all organisms, from prokaryotes to eukaryotes. Beside its predominant involvement in NTP synthesis, NDK performs a variety of functions in bacteria, such as cell growth and differentiation, transcription regulation, site-specific DNA cleavage, and signal transduction (Biggs et al., 1990; Choi et al., 1999; Postel et al., 2000; Steeg et al., 2003). In recent years, extracellular secretion of NDK has been reported in several organisms, including pathogens such as Leishmania amazonensis (Kolli et al., 2008), M. tuberculosis (Chopra et al., 2003), P. gingivalis (Yilmaz et al., 2008), Pseudomonas aeruginosa (Zaborina et al., 2000), T. spiralis (Gounaris et al., 2001) and V. cholerae (Punj et al., 2000). In fact, the involvement of secretory M. tuberculosis NDK (mNDK) in phagosome maturation has been shown recently (Sun et al., 2010). However, there is no report available about the secretory nature of Salmonella enterica serovar Typhimurium (S. Typhimurium) NDK (sNDK), which was described as early as 1974 (Ginther & Ingraham, 1974).

Despite being a well-known enzyme, several fundamental questions about bacterial NDKs are still unanswered. In an effort towards understanding this protein in greater detail, we have carried out biochemical characterization of NDKs from intracellular pathogens, M. tuberculosis (Tiwari et al., 2004) and S. Typhimurium (Dar & Chakraborti, 2010). In this study, the recognition of a concentrated cell-free supernatant of S. Typhimurium by anti-mNDK antibody and its ability to synthesize NTPs established the secretory nature of this enzyme. Since NDK utilizes NTPs as its substrate, its secretion from pathogens could be important in the context of eATP metabolism (Dubyk & el-Moatassim, 1993; Ferrari et al., 1997; Zaborina et al., 1999a). We therefore assessed the contribution of secretory NDKs from intracellular pathogens, M. tuberculosis and S. Typhimurium and extracellular (V. cholerae) pathogens to the process of ATP-induced J774 mouse macrophage cell lysis by monitoring lactate dehydrogenase (LDH) release in the culture medium. We show here that the secretory NDK from intracellular pathogens is able to prevent ATP-induced J774 mouse macrophage cell lysis. On the other hand, in an extracellular pathogen such as V. cholerae, the concentrated culture supernatant containing V. cholerae NDK (vNDK) exhibited a cytotoxic effect on J774 cells. However, the secretory vNDK obtained following its expression in an ndk-deleted strain of S. Typhimurium (Δndk) displayed behaviour similar to that of the intracellular pathogens. Thus, our results for what is believed to be the first time provide evidence that secretory NDK possesses ATP-sequestration ability irrespective of its extra- or intracellular mode of manifestation.

METHODS

Materials. Restriction and modifying enzymes were obtained from New England Biolabs. All fine chemicals, including different nucleotides (ATP, UTP, GTP), oxidized ATP (O-ATP) and benzyl-benzyl ATP (Bz-ATP), were procured from Sigma. The oligonucleotides used in this study were custom-synthesized (Integrated DNA Technologies/Sigma). [γ-32P]ATP (~5000 Ci mmol⁻¹; 185 TBq mmol⁻¹) was purchased from Jolanki Laboratories, Board of Radiation and Isotope Technology, Hyderabad, India. Polyethyleneimine (PEI) Cellulose F plates were procured from Merck.

Bacterial strains. S. Typhimurium (clinical isolate; Mukherjee et al., 2000), V. cholerae (strain N16961) and M. tuberculosis (strain H37Ra) were used in this study. Unless mentioned otherwise, all bacterial cultures were grown at 37 °C at ~200 r.p.m. in a rotary shaker (Innova 4230, New Brunswick Scientific). For S. Typhimurium and V. cholerae, Luria–Bertani (LB) broth (Sambrook & Russell, 2001), and for M. tuberculosis, Middlebrook 7H9 medium, were used. The bacterial secretory products were prepared as described in earlier studies (Melnikov et al., 2000; Punj et al., 2000; Zaborina et al., 1999b), with minor modifications. Briefly, overnight cultures of S. Typhimurium and V. cholerae were reincubated at OD₆₀₀ 0.01, grown for 4 h to exponential phase (OD₆₀₀ 0.4–0.5), harvested and filtered through a 0.22 µm pore-size filter (Millex-HV, Millipore). Bacterial cells obtained as pellets were, if necessary, resuspended in 50 mM Tris, pH 7.5, containing 150 mM NaCl, and following sonication, the supernatant fraction (also termed ‘lysate’) was used for determination of intracellular LDH activity. For lag and stationary phase samples of S. Typhimurium, cultures were grown in LB to OD₆₀₀ 0.1 and 1.05, respectively. For studies in different media, S. Typhimurium was grown in TB (Bacto tryptone, Bacto beef extract), TYE (Bacto tryptone, yeast extract) and M9 (glucose, casamino acids) media to exponential phase (Melnikov et al., 2000). In the case of M. tuberculosis, cultures were harvested after 10–12 days of growth. All samples (S. Typhimurium, M. tuberculosis and V. cholerae secretory products) were further concentrated using 10 kDa cut-off concentrators (Amicon) and were confirmed to be free from bacterial contamination.

DNA manipulations. The construction of pET-sNDK (S. Typhimurium ndk cloned into the Ndel/HindIII sites of pET-28c) has been described previously (Dar & Chakraborti, 2010). V. cholerae ndk (vndk; locus tagVCO756; Heidelberg et al., 2000) was amplified by PCR from genomic DNA using gene-specific primers (CH3 and CH4, Supplementary Table S1) and cloned into pUC19 at the Smal site to generate pUC-vNDK. This construct was digested with Ndel/HindIII and ligated at the corresponding sites of pET-28c to generate pET-vNDK for use in obtaining the recombinant protein (vNDK). A PCR-based overlap-extension method was employed for generation of conserved histidine (H117A) point mutations in both sNDK and vNDK. For this, two external and two internal (incorporating mutation) primers (Supplementary Table S1) were designed. Two sets of primary (pET-sNDK/pET-vNDK as the template) and one set of secondary (mixture of primary reaction products as the template) PCRs were carried out for each mutation. All constructs (wild-type or mutant) were individually transformed into Escherichia coli strains DH5α and BL21(DE3) to increase the amount of DNA and the expression of recombinant proteins, respectively. All mutations were confirmed by sequencing.

Expression and purification of recombinant proteins. E. coli BL21(DE3) cells harbouring pET-sNDK, pET-vNDK or histidine mutant constructs were grown overnight in LB broth containing kanamycin (50 µg ml⁻¹). A fresh culture was then set up with 1 % inoculum (from an overnight culture), grown to OD₆₀₀ 0.6 and then induced with 0.4 mM IPTG. Cells after 12 h incubation at 18 °C were harvested, resuspended in lysis buffer [50 mM Tris, pH 7.5, containing 150 mM NaCl, 1 mM PMSF, 1 µg ml⁻¹ each of pepstatin and leupeptin or inhibitor cocktail (Roche)], treated with lysozyme (30 min at 4 °C) and sonicated. The supernatant fraction was further
loaded onto a nickel-nitriolate (Ni-NTA) column and washed (10 bed volumes with 50 mM Tris, pH 7.5, containing 150 mM NaCl and 10 mM imidazole), and the purified protein(s) was eluted with elution buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 150 mM imidazole). Following removal of imidazole by dialysis (Dar & Chakraborti, 2010), protein was estimated (Bradford, 1976) and the sample was stored at –80 °C until use.

**Construction of ndk knockout strains.** The ndk knockout strains of *S. Typhimurium* (Δndk) and *V. cholerae* (Δndk) were constructed employing the bacterial conjugation protocol of Miller & Mekalanos (1988). For this purpose, an internal 250 bp fragment of the *ndk* or *vndk* gene was PCR-amplified using pET-sNDK or pET-vNDK as a template with a set of primers (Supplementary Table S1) incorporating EcoRI/XhoI sites, and was cloned at the corresponding sites of the pGPF704 vector. The construct (pGPF704-NDK250 or pGPF704-vNDK250), following transformation into *E. coli* SM10/pir (Miller & Mekalanos, 1988; Raychaudhuri et al., 2006), yielded the donor strain. Cultures of the donor and recipient (wild-type S. Typhimurium and *V. cholerae*) strains (both OD600 0.4 in LB broth) were mixed, spotted onto an LB agar plate and incubated (16–20 h). The plate was scraped, inoculated into fresh LB broth supplemented with antibiotics (streptomycin and ampicillin, both 100 µg ml⁻¹) and kept static overnight (37 °C) following vigorous mixing. The culture (upper portion) was plated on an LB agar plate containing the appropriate antibiotics. Colonies were grown for three to four generations, and the *ndk*-deleted strain(s) was confirmed by PCR (data not shown) as well as by Western blotting utilizing anti-mNDK antibody.

pGEX-Kan was constructed by inserting a kanamycin cassette at the *PstI* site of the ampicillin-resistance gene of vector pGEX-KG, and was used for expression of vNDK in Δndk. For this, pGEX-Kan and pUC-vNDK were digested with *BamHI* and *NdeI*, respectively, treated with Klenow and then digested with *SfiI*. The insert and vector following ligation were transformed into DH5α and selected on LB-kanamycin plates to obtain pGEX-Kan-vNDK. pGEX-Kan-vNDK was transformed into electrocompetent Δndk cells, and the transformants were selected on LB plates with streptomycin, ampicillin and kanamycin (100, 50 and 50 µg ml⁻¹, respectively). An overnight culture from a single colony was inoculated in fresh medium, grown to OD600 0.5–0.6, induced with 0.4 mM IPTG (3 h incubation), harvested and processed further. Secretory products from Δndk, Δvndk and Δndk transformed with *ndk* of *V. cholerae* or *S. Typhimurium* were prepared as described above (Methods, Bacterial strains).

**Autophosphorylation and phosphotransfer assays.** The autophosphorylation abilities of sNDK, vNDK and histidine mutants were assessed as described previously (Dar & Chakraborti, 2010; Tiwari et al., 2004). Purified protein (500 ng per reaction) was incubated in 1× kinase buffer with 2 µCi (74 kBq) [γ-32P]ATP at 24 °C for 1 min, and reaction products were resolved on a 12% SDS-PAGE gel. The labelled bands were analysed with a phosphoimager (FLA-9000, Fujiﬁlms) as well as by autoradiography.

The NTP-synthesizing activities of sNDK, vNDK and histidine mutants were monitored in a colorimetric assay performed in microtitre plates (Dar & Chakraborti, 2010; Lascu et al., 1993; Tiwari et al., 2004). Briefly, the enzyme activity of samples (20 ng sNDK or 150 ng vNDK), following incubation with substrate (5–400 µM ADP) and other reaction ingredients (reaction volume=50 µl), was monitored at 490 nm in an ELISA plate reader (SpectraMax Plus 384, Molecular Devices). The values obtained were corrected by subtracting the blank readings (no significant difference was noticed when assays were carried out with all ingredients, except either substrate or sNDK and dGTP). Standard curves were prepared with ATP (5–200 µM; omitting dGTP, ADP and sNDK or vNDK in reaction mixtures), and the activity of sNDK/vNDK was expressed as µmole ATP produced min⁻¹ (mg protein)⁻¹.

To evaluate the NDK activity in the cell-free supernatant, a kinase assay was performed using PEI TLI plates (PEI-TLI Cellulose F plates, 1.05579.0001, Merck). Briefly, 5 µl concentrated cell-free supernatant containing 5 µg total protein was incubated with 2 µCi (74 kBq) [γ-32P]ATP, 1 mM NDP or a mixture of NDPs and 1 mM MgCl₂ (Melnikov et al., 2000; Kolli et al., 2008). The reaction was performed at room temperature for 10 min and then stopped by incubating at 4 °C (over ice). Finally, 2.5 µl of the reaction product was separated on a PEI-TLI plate using 0.75 M KH₂PO₄ buffer (pH 3.5). The plate was air-dried, developed and analysed on a phosphoimager as well as by autoradiography. For adenylate kinase activity, the concentrated cell-free supernatant was incubated with [γ-32P]ATP in the presence of 1 mM AMP (Melnikov et al., 2000; Kolli et al., 2008).

**Macrophage culture and LDH assay.** 1774 mouse macrophage cells (3–5×10⁴) in 10 ml RPMI medium with 10% (v/v) fetal calf serum (FCS) were cultured in a 90 mm tissue culture plate at 37 °C in a CO₂ incubator (95% O₂/5% CO₂) for 16–18 h. Cells were harvested (2000 g for 2–3 min at 4 °C), washed three times with chilled PBS and then counted using the trypan blue method. Cells (2×10⁵ per well) in RPMI with 3% FCS were seeded in 24-well plates and incubated at 37 °C for 2–3 h to test adherence. Following washing with PBS, adhered cells were incubated in RPMI/10% FCS with ATP or concentrated cell-free filtrate containing bacterial secretory products (S. Typhimurium, *M. tuberculosis*, *V. cholerae*, Δndk, Δvndk or Δndk transformed with *ndk* of *V. cholerae* or *S. Typhimurium*) with or without ATP for 4 h (Melnikov et al., 2000; Kolli et al., 2008; Zaborina et al., 1999b). In all cases the effect of bacterial secretory products was evaluated using 1, 5 or 10 µg total protein (in 10 µl), except for *M. tuberculosis*, where 1, 5 or 10 µl of concentrated sample (processed similarly) was used. At the end of incubation, the supernatant (50 µl) was removed and the LDH activity (of the supernatant) was colorimetrically determined with a CytoTox 96(R) Non-Radioactive Cytotoxicity Assay kit, following the manufacturer’s protocol (Promega). The LDH activity obtained in the supernatant (50 µl) of cells without any treatment served as a blank. The blank value was subtracted in each case, and the percentage LDH release was calculated taking 0.2% Triton X-100-lysed cells as 100% (Kolli et al., 2008).

**Western blotting.** Samples (~10 µg per slot) of wild-type (*S. Typhimurium* or *V. cholerae*), their ndk-deleted strains, or concentrated cell-free secretory products, after resolving by 12% SDS-PAGE, were transferred (120 V for 1 h) to a nitrocellulose membrane (0.45 µm) using Tris-glycine-SDS buffer (Dar & Chakraborti, 2010). Blots were incubated in primary (anti-mNDK) and secondary (anti-rabbit IgG) antibodies, or horseradish peroxidase-conjugated anti-glutathione S-transferase (GST) antibody, and developed with the ECL detection system (GE Healthcare) following the manufacturer’s recommended protocol.

**Bioinformatic analysis.** The multiple sequence alignment of the protein sequences retrieved from the mail server at the NIH, USA (http://www.ncbi.nlm.nih.gov), was carried out using the CLUSTAL_X 1.81 program (Thompson et al., 1997).

**Statistical analysis.** Data were analysed with Student’s t test, the non-parametric Mann–Whitney two-tailed test and repeated-measure analysis of variance with the Student–Newman–Keuls multiple-comparisons post test using Instat software (version 3, GraphPad).

**RESULTS**

sNDK is secretory in nature

NDKs from different bacteria have been shown to be secretory in nature (Chopra et al., 2003; Punj et al., 2000;
Yilmaz et al., 2008; Zaborina et al., 2000). To elucidate this aspect in S. Typhimurium, concentrated culture supernatant from different phases of bacterial growth was incubated with NDPs (CDP, GDP and UDP) in the presence of [$\gamma$-32P]ATP, and the reaction products were resolved on TLC plates (see Methods). This led to the formation of the corresponding NTPs (CTP, GTP and UTP) in the exponential phase of the culture (Fig. 1a, left panel and Supplementary Fig. S1a), suggesting NDK-like activity in the samples. Among the different culture media tested (LB, TB, TYE and M9), such an activity could not be observed in M9 medium (Supplementary Fig. S1b). In subsequent studies, we used exponential phase cultures of S. Typhimurium grown in LB medium. The same experiment, when carried out with concentrated culture supernatant of M. tuberculosis, yielded identical results (Fig. 1a, right panel, compare lane 1 with lanes 2, 3 and 4). We carried out Western blotting using anti-mNDK antibody (Tiwari et al., 2004). As shown in Fig. 1(b), this antibody was able to recognize sNDK as well (upper panel, lane 2). Further, the cell lysate and concentrated cell-free supernatant prepared from exponential phase culture (see Methods) of S. Typhimurium were resolved by SDS-PAGE and subjected to immunoblotting using anti-mNDK antibody. Interestingly, in both cell lysate and concentrated cell-free supernatant of S. Typhimurium, bands corresponding to the expected size of NDK (~16.5 kDa) were highlighted (Fig. 1c, upper panel, lane 3). To rule out the possibility of any bacterial contamination, the concentrated cell-free supernatant was spotted onto LB agar without any antibiotic selection. As expected, the concentrated cell-free supernatant did not show such contamination (Fig. 1d, upper panel, lane 3). To make sure that the presence of NDK in concentrated cell-free supernatant was not due to cell lysis, we determined its LDH activity. LDH is a known cytoplasmic enzyme and is used as a marker for cell lysis. As shown in Fig. 1(d) (lower panel), only cell lysate (supernatant prepared from sonicated cells) exhibited LDH activity [compare panels of lysate versus concentrated cell-free supernatant and also concentrated medium (inset)]. Thus our results argue that the secretory product of S. Typhimurium very likely contains NDK.

Since NDK utilizes ATP as its substrate, its secretion from pathogens could be important in the context of ATP sequestration, and as a result it should prevent cytolsis (Gounaris et al., 2001; Kolli et al., 2008; Yilmaz et al., 2008). Based on this hypothesis, we utilized an assay system in which the cytotoxic effect or behaviour of ATP was studied by monitoring the release of the cytoplasmic marker enzyme LDH from J774 cells (Kolli et al., 2008; Melnikov et al., 2000; Punj et al., 2000). The system displayed ATP-dependent release of LDH from J774 cells (Fig. 2a, compare first three bars), and this effect was ATP-specific, as treatment with either GTP or UTP did not cause any LDH release (Fig. 2a and inset, showing that the percentage LDH release was equal to that from cells without any treatment). To test whether ATP-induced LDH release is mediated only through P2X7 receptors, J774 macrophage cells were pre-treated with a known receptor-specific antagonist and an antagonist (Melnikov et al., 2000; Punj et al., 2000; Yilmaz et al., 2008). As expected, while pre-treatment (1 h) with the irreversible antagonist O-ATP (0.3 mM) prevented ATP-induced LDH release, an agonist, Bz-ATP (2 mM), did not affect such activity (Fig. 2a, compare last two bars). In our experimental conditions, we observed ~60% LDH release in response to ATP treatment of J774 macrophage cells, which is well within the range (20–75%) reported by others (Kolli et al., 2008; Melnikov et al., 2000). Compared with treatment with ATP alone, incubation of concentrated cell-free supernatants from either M. tuberculosis or S. Typhimurium with adhered J774 cells in the presence of ATP prevented LDH release in a dose-dependent manner (Fig. 2b). This was an ATP-dependent phenomenon, since the percentage LDH release in response to concentrated cell-free supernatants from either M. tuberculosis or S. Typhimurium was similar to that of the cells without any treatment (inset of Fig. 2b). Since 10× concentrated cell-free supernatants displayed maximum prevention of ATP-induced J774 cell death (in the presence of 3 mM ATP, percentage LDH release with S. Typhimurium was 17.98 ± 3.94 and with M. tuberculosis = 23.7 ± 4.88, as opposed to 58.9 ± 5.45 with ATP alone; n=5 in all cases; P<0.001; Fig. 2b), in subsequent assays this amount was used. Thus our results indicated that secretory ATP-utilizing enzymes, including NDK of intracellular pathogens such as M. tuberculosis and S. Typhimurium, could prevent the ATP-induced cytotoxicity of J774 macrophage cells.

We further constructed an ndk knockout strain of S. Typhimurium (Δsndk) employing a bacterial conjugation method (see Methods and Supplementary Fig. S2a). The deletion of NDK was confirmed by Western blotting using anti-mNDK antibody (Supplementary Fig. S2b). To substantiate that the NTP-synthesizing activity of the cell-free supernatant of S. Typhimurium was due to the presence of NDK, the secretory product from the Δsndk strain was incubated with [γ-32P]ATP and a mixture of NDPs. Fig. 3(a) categorically demonstrates that the secretory product (concentrated) obtained from the culture of the Δsndk strain was unable to synthesize any of the NTPs compared with that of the wild-type (left panel, lane 3). Purified protein (sNDK) was included as a positive control in this experiment, which on incubation with mixture of NDPs (CDP, GDP and UDP) efficiently converted them to the corresponding NTPs (Fig. 3a, left panel, lane 4). Interestingly, concentrated cell-free supernatant from an ndk-deleted strain of S. Typhimurium on incubation with [γ-32P]ATP and AMP was able to form labelled ADP but unable to synthesize ATP, suggesting that deletion of NDK did not affect the adenylate kinase-like activity of the secretory product from the pathogen (Fig. 3a, right panel, compare lanes 2 and 3). The formation of labelled products by the Δsndk strain in the presence of [γ-32P]ATP (Fig. 3a, left panel, lane 3) may be due to the
combined action of adenylate kinase and 5'-nucleotidase enzyme activities present in the cell-free supernatant of the ndk-deleted strain of *S.* Typhimurium. Similar behaviour has been reported earlier in *Burkholderia cepacia* (Melnikov et al., 2000) and *Pseudomonas aeruginosa* (Zaborina et al., 1999b), in which in the absence of any substrate, the incubation of the secreted product with [$\gamma^{-32}$P]ATP results in the formation of labelled products, including ADP.

We also tested the effect of deletion of *ndk* on the ATP-scavenging ability of *S.* Typhimurium concentrated culture supernatant. For this, J774 macrophage cells were treated
with concentrated cell-free supernatant of the Δnsdk strain, alone or in the presence of ATP, and cell lysis was assessed by monitoring LDH release. Interestingly, we found that the ATP was able to cause significant (P<0.001) J774 cytotoxicity when incubated with concentrated culture supernatant from the Δnsdk strain (percentage LDH release in Δnsdk + ATP = 41 ± 6.2, n = 3; Fig. 3b). As expected, the percentage LDH release with the Δnsdk strain was very close to that of cells without any treatment (percentage LDH release in Δnsdk = 19.2 ± 3.2, as opposed to 18.5 ± 3.6 for cells alone; n = 3 in each case; inset of Fig. 3b). Thus these results indicate that the prevention or modulation of ATP-induced cell lysis by secretory ATP-utilizing enzymes of S. Typhimurium is mediated through NDK.

**Secretory vNDK is cytotoxic to J774 mouse macrophage cells**

To have an insight into the secretory nature of the NDK of the extracellular pathogen V. cholerae, cell lysate and concentrated culture supernatant were prepared from exponential phase cultures (see Methods). Samples, after resolving by SDS-PAGE, were subjected to Western blotting using anti-mNDK antibody. The antibody bound to bands at ~15.8 kDa (Fig. 4a), which was the expected size of vNDK. TLC of the concentrated culture supernatant upon incubation with different NDPs in the presence of [γ-32P]ATP yielded the corresponding NTP, which was suggestive of an NDK-like activity in the samples (Fig. 4b). Further, we observed that incubation of concentrated cell-free supernatants from V. cholerae with adhered J774 cells in the presence of ATP did not prevent LDH release. In fact, there was hardly any difference in the level of LDH released in the presence of ATP alone (percentage LDH release 56.8 ± 7.7, n = 3) or ATP and secretory product (65.3 ± 4.7, n = 3; Fig. 4c). V. cholerae secretory product containing NDK (vNDK), even in the absence of ATP, displayed a cytotoxic effect (percentage LDH release 63.25 ± 6.7, n = 3; Fig. 4c). This was not an experimental artefact, since the cytotoxicity rendered by concentrated cell-free supernatants of V. cholerae increased in a

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*Fig. 2. Role of secretory NDK in ATP-induced J774 macrophage cell lysis. (a) Effect of ATP on J774 cells. Macrophage cells were treated with different concentrations of ATP (1, 3 and 5 mM) or different NTPs (GTP, UTP; 3 mM each) or pre-treated for 1 h with O-ATP (0.3 mM) or Bz-ATP (2 mM), and then incubated with ATP (3 mM) for 4 h as indicated. Samples were collected as described in the text and analysed for LDH release. The amount of LDH released by cells alone served as a blank and was subtracted from each sample. Inset: LDH release by different NTPs (3 mM) compared with that due to cells alone. (b) Effect of secretory NDKs from intracellular pathogens on the modulation of ATP-induced cell lysis. Macrophage cells were incubated with buffer or ATP for 4 h in the presence or absence of different doses (1×, 5× and 10×) of concentrated cell-free supernatant from S. Typhimurium (S.t) or M. tuberculosis (M.t) as indicated, and an LDH release assay was performed. Percentage LDH release was calculated taking 0.2 % Triton X-100-lysed cells as 100%. Cells alone served as a blank and this value was subtracted from test samples. Data represent mean and SD (n = 3 or 5). ** and *** indicate P<0.01 and P<0.001, respectively; 1×, 5× and 10× represent 1, 5 and 10 μg for S. Typhimurium, and 1, 5 and 10 μl for M. tuberculosis. Inset: LDH release from cells in response to no treatment (cells only) or treatment with 10× concentrated culture supernatant from S. Typhimurium (S.t) or M. tuberculosis (M.t) in the absence of ATP.
dose-dependent manner (inset, Fig. 4c, compare LDH release of 1 × and 10 × concentrated culture supernatant). Thus our results are in conformity with those of others (Punj et al., 2000; Zaborina et al., 2000) and support the notion that secretory products containing ATP-utilizing enzymes, including NDKs of extracellular pathogens, are cytotoxic to macrophages. It is interesting to mention here that the secretory product containing ATP-utilizing enzymes (including NDK) of a V. cholerae toxin-deficient strain (V. cholerae VB1) exhibits similar behaviour (Punj et al., 2000).

**NDKs from S. Typhimurium and V. cholerae exhibit identical biochemical characteristics**

NDK, one of the secretory ATP-utilizing enzymes, is involved in preventing ATP-induced cell lysis (Fig. 2) in intracellular pathogens (S. Typhimurium and M. tuberculosis), while its extracellular counterpart (V. cholerae) is cytotoxic to J774 mouse macrophage cells (Fig. 4). Any alteration in protein functionality is likely to be reflected in the sequence. Interestingly, a pairwise sequence comparison indicated that sNDK and vNDK share 65% identity (Supplementary Fig. S3a). NDK is known to maintain the NTP/dNTP pools of a cell by synthesizing different NTPs/dNTPs through a high-energy phosphorylated intermediate (Lacombe et al., 2000; Lascu, 2000). Both sNDK and vNDK recombinant proteins were found to display autophosphorylation as well as phosphotransfer activities (Fig. 5a, b). NDK is known to be a histidine kinase, in which a highly conserved active site histidine residue is indispensable for the activity of the protein. To determine the effect of a mutation on the activities of the proteins, His-117 was mutated to alanine, and the protein was then assessed for autophosphorylation and phosphotransfer ability. The results demonstrated that the mutation abolished both the activities of the recombinant proteins, making them kinetically inactive (Fig. 5a, b). Kinetically these proteins also showed minor differences, e.g. the enzyme turnover rate of sNDK was slightly higher compared with vNDK (Table 1). Interestingly, recombinant NDK from another intracellular pathogen, M. tuberculosis, exhibited an approximately ninefold increase in enzyme turnover rate compared with vNDK (Tiwari et al., 2004).

**Secretory NDKs from S. Typhimurium and V. cholerae exhibit identical behaviour**

Despite their highly homologous amino acid sequences and similar biochemical properties (Fig. 5), the distinction in the functionality of secretory NDKs from extra- or intracellular pathogens in preventing ATP-mediated cytotoxicity (Figs 2 and 4) was surprising. To account for the bifunctional nature of this secretory protein, we expressed NDK from V. cholerae in an ndk-deleted strain of S. Typhimurium. Fig. 6(a) clearly shows the expression of an ~44 kDa induced band, which corresponds to the expected size of the protein of interest (compare lanes 1–5 with lane 6), and its authenticity as vNDK was confirmed by Western blotting using anti-mNDK (Fig. 6a, middle panel) and anti-GST (Fig. 6a, lower panel) antibodies.
To test whether the expression of NDK from an extracellular pathogen \( (V. cholerae) \) could rescue the NTP-synthesizing deficiency of an \( ndk \)-deleted strain of \( S. Typhimurium \), concentrated culture supernatant from a vNDK-transformed \( \Delta ndk \) strain was prepared and analysed for NTP synthesis. The results obtained clearly demonstrated that expression of NDK from an extracellular pathogen can complement the function of an intracellular pathogen by restoring the NTP-synthesizing ability of the \( \Delta ndk \) strain (Fig. 6b, compare lanes 4 and 5 with lanes 6 and 7). As expected, the vector transformed in \( \Delta ndk \) was unable to synthesize any NTP (Fig. 6b, compare lanes 6 and 7 with lanes 8 and 9). We also monitored LDH release following incubation of concentrated culture supernatants (10 \( \mu \)g protein) of the \( \Delta ndk \) strain, complemented with either vNDK or sNDK, with J774 cells in the presence of ATP. Surprisingly, we observed that incubation with concentrated culture supernatant from the vNDK-complemented \( \Delta ndk \) strain was able to prevent ATP-induced LDH release from J774 cells (21.9 \( \pm \) 4.05 %, compared with 55.85 \( \pm \) 2.0 % with ATP alone, \( n=5 \); Fig. 6(c), compare bars 1 and 4). In fact, the behaviour of the wild-type strain of \( S. Typhimurium \) and the \( \Delta ndk \) strain complemented with either sNDK or vNDK was indistinguishable in the presence (Fig. 6c, compare bars 2 and 3 with bar 4) or absence (inset, Fig. 6c) of ATP. Furthermore, the concentrated secretory product from the \( ndk \)-deleted strain of \( V. cholerae \), when incubated with J774 cells, exhibited cytotoxic behaviour even in the absence of ATP (Fig. 6c). Thus all these lines of evidence unambiguously rule out the
possibility of any distinction in the function of NDKs between extracellular and intracellular pathogens.

**DISCUSSION**

NDK is a highly conserved ubiquitous enzyme primarily responsible for maintaining nucleotide pools in microorganisms. Autophosphorylation of the protein occurs as a part of its catalytic mechanism, which leads to the phosphotransfer reaction from NTP/dNTP to NDP through a high-energy phosphorylated intermediate (Lacombe et al., 2000). Since NDK has very little specificity for the base or the sugar, it has the ability to accept any NTP/dNTP as its substrate. A growing body of evidence now indicates that NDKs, as well as other ATP-utilizing enzymes of microbes, especially in pathogens, are secretory in nature and involved in host–pathogen interactions. In fact, host cells as a part of their defence strategy, when infected by pathogens, accumulate eATP at the site of inflammation, which causes cell death (Dubyak & el-Moatassim, 1993; Ferrari et al., 1997). It has now been postulated that secretory NDK or other ATP-utilizing enzymes from pathogens modulate the level of eATP and thus exploit the situation in favour of their survival (Chopra et al., 2003; Gounaris et al., 2001; Yilmaz et al., 2008; Zaborina et al., 1999a). However, the distinct physiological requirements of intra- and extracellular pathogens complicate the situation slightly. This led to the hypothesis that intra- and extracellular pathogens use secreted ATP-utilizing enzymes in opposite directions, by either avoiding or promoting the phagocytic activity of macrophages and/or other professional phagocytes (Kolli et al., 2008; Punj et al., 2000; Yilmaz et al., 2008; Zaborina et al., 2000). To rationalize the notion, we carried out our studies with intracellular (S. Typhimurium) and extracellular (V. cholerae) pathogens to explore the behaviour of secretory NDK.

NDK is reported to be the only enzyme involved in the maintenance of NTP pools in S. Typhimurium (Ginther & Ingraham, 1974; Ingraham & Ginther, 1978). Several lines of evidence in our study unambiguously established the secretory nature of this enzyme in S. Typhimurium (Fig. 1 and Supplementary Fig. S2) and thus corroborated reports for other pathogenic microbes (Coutinho-Silva et al., 2001; Gounaris et al., 2001; Punj et al., 2000; Zaborina et al.,...
We further ascertained that the concentrated culture supernatant of this intracellular pathogen could prevent ATP-induced LDH release (Fig. 2b), while its extracellular counterpart from *V. cholerae* exhibited cytotoxic behaviour (Fig. 4c). Considering that NDKs from *S. Typhimurium* and *V. cholerae* show 65% identity at the amino acid level (Supplementary Fig. S3), that both are secretory (Figs 1 and 4a) and that they are able to utilize ATP as substrate (Fig. 5a, b), their bifunctional nature (Chopra et al., 2003; Punj et al., 2000; Yilmaz et al., 2008) is quite puzzling.

To resolve this issue, we exploited a Δ*sndk* strain, in which other ATP-utilizing enzymes secreted by *S. Typhimurium* are unable to carry out the function of NDK and are unable to modulate the ATP-induced cytotoxicity of J774 cells (Fig. 3b). Since the biochemical characteristics of NDKs from intra- and extracellular pathogens are similar, we wanted to determine whether the deficiencies of the Δ*sndk* strain could be functionally complemented by vNDK. To our surprise, we found that the concentrated culture supernatant of the vNDK-transformed Δ*sndk* strain could synthesize NTPs and was also able to prevent the ATP-induced cytotoxicity of J774 cells (Fig. 6b, c). To address the discrepancy in the behaviour of secretory vNDK from wild-type *V. cholerae* (Fig. 4b) and from the vNDK-transformed Δ*sndk* strain with respect to the prevention/modulation of ATP-induced J774 macrophage cell lysis (Fig. 6c), we further generated an ndk-deleted strain of *V. cholerae* (Δndk). The concentrated culture supernatant from this strain, when incubated with J774 cells, exhibited the same level of cell killing as that observed in the presence of ATP alone (Fig. 6c), suggesting that secretory NDK from an extra-
cellular pathogen per se does not contribute to the cytotoxicity of mouse macrophage cells. Thus our findings address a long-standing unresolved issue concerning the pathogen-specific bifunctional behaviour of NDK, and certainly demand a revisit of the traditional concept.

NDKs from intra- and extracellular pathogens might have different roles to play, despite being biochemically indistinguishable. This is very likely because of their distinct physiological niches. In fact, pathogens are known to secrete different proteins, factors or even effectors, which either regulate different processes or modulate the function of a known protein(s), and thus may render toxicity to the host cells for their survival (Paumet et al., 2009; Garcia-del Portillo & Finlay, 1995; Prost et al., 2007). Further study in this direction is necessary to illuminate such a perplexing issue. Nonetheless, our results for what we believe to be the first time lend credence to the fact that the intrinsic properties of NDKs from intra- and extracellular pathogens cannot be distinguished.

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